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The Angiotensin AT_2 Receptor: From a Binding Site to a Novel Therapeutic Target

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ABBREVIATIONS: AA, aortic aneurysm; ACE, angiotensin-converting enzyme; ACE2, angiotensin-converting enzyme-2; AD, Alzheimer's disease; AdipoR, adiponectin receptor; Akt, protein kinase B; ALI, acute lung injury; Ang IV, angiotensin IV; Ang-(1-7), angiotensin-(1-7); Ang-(1-9), angiotensin-(1-9); APA, aminopeptidase A; ApoE, Apolipoprotein E; AQP4, aquaporin-4 water channel; ARB, AT_1R blocker; ATIP, AT_2R-interacting protein; AT_1R, angiotensin AT_1 receptor; AT_2R, angiotensin AT_2 receptor; AT_2R-KO, AT_2R-knockout mice; ATTRACT, Angiotensin II Type Two Receptor Agonist in COVID-19 Trial; AVP, vasopressin; BAT, brown adipose tissue; BBB, blood-brain barrier; BDNF, brain-derived neurotrophic factor; BK, bradykinin; BP, blood pressure; B_2R, bradykinin B_2 receptor; BRIL, b_{562}RIL; C21, N-[[3-[4-(1H-imidazol-1-ylmethyl)phenyl]-5-(2-methylpropyl)-2 thienyl]sulfonyl]carbamic acid butyl ester (IUPAC/chemical name); C38, N-[[3-[3-(1H-imidazol-1-ylmethyl)phenyl]-5-(2-methylpropyl)-2 thienyl]sulfonyl]carbamic acid butyl ester (IUPAC/chemical name); candesartan, 2-ethoxy-3-[[4-[2-(2H-tetrazol-5-yl)phenyl]phenyl]methyl]benzimidazole-4-carboxylic acid; CGP42112A, (2S, 3S)-2-[[(2S)-1-[(2S)-2-[[(2S)-6-[[(2S)-5-(diaminomethylideneamino)-2-(phenylmethoxycarbonylamino)pentanoyl]amino]-2-[[(2S)-3-(4-hydroxyphenyl)-2-(pyridine-3-carbonylamino)propanoyl]amino]hexanoyl]amino]-3-(3H-imidazol-4-yl)propanoyl]pyrrolidine-2-carbonyl]amino]-3-methylpentanoic acid (IUPAC name); CKD, chronic kidney disease; CNK, connector enhancer of Ksr; CNS, central nervous system; co-IP, coimmunoprecipitation; compound 1 (L-161,638), 2-ethyl-6-[N-benzyl-N-(2-thienoyl)amino-3-[[2'-(1H-tetrazol-5-yl)-[1,1']-biphenyl-4-yl]-methyl]quinazolin-4-(3H)-one; compound 2, 2-propyl-6-[N-2-furanmethylenyl -N-(benzoyl)amino-3-[[2'-(1H-tetrazol-5-yl)-[1,1']-biphenyl-4-yl]methyl]-quinazolin-4-(3H)-one; COVID-19, coronavirus disease 2019; CTGF, connective tissue growth factor; CVD, cardiovascular disease; DN, diabetic nephropathy; D_1R, dopamine D_1 receptor; DRG, dorsal root ganglia; EC, extracellular; ECFV, extracellular fluid volume; ECL1–3, extracellular loops 1–3; ECM, extracellular matrix; eGFP, enhanced GFP; EMA300 (synonym PD121981), 1H-imidazo[4,5-c]pyridine-6-carboxylic acid, 5-(diphenylacetyl)-4,5,6,7-tetrahydro-1-[(4-methoxy-3-methylphenyl)methyl]-, (S)- (9CI); EMA400 (synonym PD126055), (S)-5-(benzyloxy)-2-(2,2-diphenylacetyl)-6-methoxy-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid; EMA401 (S-enantiomer of EMA-400), (3S)-2-(2,2-diphenylacetyl)-1,2,3,4-tetrahydro-6-methoxy-5-(phenylmethoxy)-3-isoquinolinecarboxylic acid (IUPAC name); eNOS, endothelial nitric oxide synthase; ERK1/2, extracellular signal-regulated kinase 1/2; fenoldopam, 6-chloro-1-(4-hydroxyphenyl)-2,3,4,5-tetrahydro-1H-3-benzazepine-7,8-diol; FRET, fluorescence resonance energy transfer; GABA, gamma aminobutyric acid; Gi or Go, inhibitory G-protein; GPCR, G-protein–coupled receptor; GTPγS, GTP gamma S; GW9662, 2-chloro-5-nitro-N-phenylbenzamide; H8, amphipathic helix 8; HEK, human embryonic kidney; HF, heart failure; HFD, high-fat diet; IC, intracellular; ICC, islet-like cell cluster; ICL1–3, intracellular loops 1–3; I_{Kv}, delayed-rectifier K^+ current; IL-6, interleukin 6; IL-10, interleukin 10; IML, intermediolateral column; Irbesartan, 2-butyl-3-[[4-[2-(2H-tetrazol-5-yl)-phenyl]phenyl]methyl]-1,3-diazaspiro[4.4]non-1-en-4-one; K_i, inhibition constant; KO, knockout; L-162,313, butyl N-[3-[4-[(2-ethyl-5,7-dimethylimidazo[5,4-b]pyridin-3-yl)methyl]phenyl]-5-(2-methylpropyl)thiophen-2-yl]sulfonylcarbamate (IUPAC name); LDL, low-density lipoprotein; L-NAME, N(G)-nitro-L-arginine methyl ester; losartan, [2-butyl-5-chloro-3-[[4-[2-(2H-tetrazol-5-yl)phenyl]phenyl]methyl]imidazol-4-yl]methanol (IUPAC name); LVH, left ventricular hypertrophy; MAPK, mitogen-activated protein kinase; MAS, MAS1 proto-oncogene, G protein-coupled receptor; MCAO, middle cerebral artery occlusion; MCT, monocrotaline; MI, myocardial infarction; MK-2206, 8-[4-(1-aminocyclobutyl)phenyl]-9-phenyl-2H-[1,2,4]triazolo[3,4-f][1,6]naphthyridin-3-one; MKP-1, MAPK phosphatase 1; MMP, matrix metalloproteinase; MS, multiple sclerosis; MTUS-1, microtubule-associated tumor suppressor 1; NEFA, nonesterified fatty acids; NF-κB, nuclear factor-kappa B; NHE, Na^+/H^+ exchanger; NMOSD, neuromyelitis optica spectrum disorder; NO, nitric oxide; NRS, numerical rating scale; NTS, solitary tract nucleus; OIR, oxygen-induced retinopathy; olmesartan (CS-866), 4-(1-hydroxy-1-methylethyl)-2-propyl-1-[[2'-(1H-tetrazol-5-yl)[1,1'-biphenyl]-4-yl]methyl]-1H-imidazole-5-carboxylic acid (5-methyl-2-oxo-1,3-dioxol-4-yl)methyl ester; OZR, obese Zucker rat; PD, Parkinson's disease; PD123177, 1-[(4-amino-3-methylphenyl)-methyl]-5-[2,2-di(phenyl)acetyl]-6,7-dihydro-4H-imidazo[5,4-d]pyridine-6-carboxylic acid (IUPAC name); PD123319 (EMA200), (6S)-1-[(4-dimethylamino-3-methylphenyl)methyl]-5-[2,2-di(phenyl)acetyl]-6,7-dihydro-4H-imidazo[5,4-d]pyridine-6-carboxylic acid (IUPAC name); PDB, Protein Data Bank; Pf-iRBC, *Plasmodium falciparum*-infected red blood cell; PH, pulmonary hypertension; PLZF, promyelocytic zinc finger protein; PNS, peripheral nervous system; PP2A, serine-threonine protein phosphatase 2A; PPARγ, peroxisome proliferator-activated receptor-γ; PSPase, serine-threonine phosphatase; PTPase, phosphotyrosine phosphatase; PTX, pertussis toxin; PVN, paraventricular nucleus; RAS, renin-angiotensin system; RPT, renal proximal tubule; RPTC, renal proximal tubule cell; RT-PCR, reverse-transcription polymerase chain reaction; RVLM, rostral ventrolateral medulla; RXFP, relaxin family peptide receptor; Sar, sarcosine; SGLT1, sodium/glucose cotransporter-1; SHP-1, Src homology region 2 domain-containing phosphatase-1; SHR, spontaneously hypertensive rat; SHR-SP, stroke-prone spontaneously hypertensive rat; SN, substantia nigra; SNA, sympathetic nerve activity; STZ, streptozotocin; telmisartan, 2-[4-[[4-methyl-6-(1-methylbenzimidazol-2-yl)-2-propylbenzimidazol-1-yl]methyl]phenyl]benzoic acid; TGF-β, transforming growth factor-β; TM, transmembrane; 7-TM, seven transmembrane; TNF-α, tumor necrosis factor-α; UCP-1, uncoupling protein-1; UUO, unilateral ureteral obstruction; Valsartan, (2S)-3-methyl-2-[pentanoyl-[[4-[2-(2H-tetrazol-5 yl)phenyl]phenyl]methyl]amino]butanoic acid (IUPAC name); VEGF, vascular endothelial growth factor; VSMC, vascular smooth muscle cell; WAT, white adipose tissue; WL-19 (synonym PD121981), (5-diphenylacetyl-1-(4-methoxy-3-methylbenzyl)- 4,5,6,7-tetrahydro-1H-imidazo[4,5-c]-pyridine-6-carboxylic acid); XLMR, X-linked mental retardation; ZD7155, 5,7-diethyl-1-[[4-[2-(2H-tetrazol-5-yl)phenyl]phenyl]methyl]-3,4-dihydro-1,6-naphthyridin-2-one.

Abstract——**Discovered more than 30 years ago, the angiotensin AT_2 receptor (AT_2R) has evolved from a binding site with unknown function to a firmly established major effector within the protective arm of the renin-angiotensin system (RAS) and a target for new drugs in development. The AT_2R represents an endogenous protective mechanism that can be manipulated in the majority of preclinical models to alleviate lung, renal, cardiovascular, metabolic, cutaneous, and neural diseases as well as cancer. This article is a comprehensive review summarizing our current knowledge of the AT_2R, from its discovery to its position within the RAS and its overall functions. This is followed by an in-depth look at the characteristics of the AT_2R, including its structure, intracellular signaling, homo- and heterodimerization, and expression. AT_2R-selective ligands, from endogenous peptides to synthetic peptides and nonpeptide molecules that are used as research tools, are discussed. Finally, we summarize the known physiological roles of the AT_2R**

and its abundant protective effects in multiple experimental disease models and expound on AT_2R ligands that are undergoing development for clinical use. The present review highlights the controversial aspects and gaps in our knowledge of this receptor and illuminates future perspectives for AT_2 R research.

***Significance Statement*——The angiotensin AT_2 receptor (AT_2R) is now regarded as a fully functional and important component of the renin-angiotensin system, with the potential of exerting protective actions in a variety of diseases. This review provides an in-depth view of the AT_2R, which has progressed from being an enigma to becoming a therapeutic target.**

I. Introduction

The identification in 1989 of two major subtypes of angiotensin receptors ushered in a new era of investigation into the renin-angiotensin system (RAS). The previously well established roles of angiotensin II (Ang II) in regulating fluid balance and the cardiovascular system were shown to be mediated by the angiotensin AT_1 receptor (AT_1R). In contrast, the angiotensin AT_2 receptor (AT_2R) was an enigma. Although radioligand binding analysis demonstrated that Ang II binds to the AT_2R with high affinity, no discernable cellular or whole-body actions of these receptors were immediately obvious. Combined with this, the early discovery that AT_2Rs are expressed in high levels in neonate tissues but decline in adults led to skepticism of their functional importance in mature individuals. This view of the AT_2R has changed over the ensuing three decades. The classification as a member of the seven transmembrane (7-TM) or G-protein–coupled receptor (GPCR) class of receptors and the discovery of intracellular signaling pathways and cellular actions that are unique to the AT_2R and distinct from that of the AT_1R led to its recognition as a functional receptor and not simply a binding site. The identification of whole-body physiological functions of AT_2R has been more elusive, although several actions in healthy animals are now established. Where the AT_2R has come to the fore is under pathophysiological conditions. AT_2R expression is increased in a multitude of disease states and its selective activation exerts protective, anti-disease actions in the vast majority of cases, often in opposition to the deleterious effects of AT_1R overactivation. As such, it is now recognized that the AT_2R is a primary component of the alternative or protective arm of the RAS. Indeed, attempts have been made to exploit AT_2R-mediated effects in various disease states to the extent that a number of AT_2R ligands have entered the drug development process and proceeded through phase I and phase II clinical trials.

The overall goal of this article is to provide a comprehensive review of the AT_2R from its discovery to its potential therapeutic application(s), with a view to convincing the biomedical field that this receptor is an important functional component of the RAS under normal physiological conditions and can be leveraged in various disease states to develop novel therapeutic approaches.

II. The Classic and the Protective Renin-Angiotensin System

A. Current View of the RAS and Discovery of AT_2R

The RAS is one of the oldest known hormonal systems that has been preserved in multiple species over millions of years (Fournier et al., 2012). The classic physiological function of the RAS is to defend the body against decreases in extracellular fluid volume (ECFV). A reduction in ECFV, either due to reduced blood volume or Na^+ depletion, results in production of Ang II via initiation of the RAS cascade (Fig. 1). Essentially, in response to the reduced ECFV, the aspartyl protease renin is released from the juxtaglomerular cells of the kidney into the circulation, where it cleaves the decapeptide angiotensin I (Ang I) from the N-terminal end of angiotensinogen, a liver $\alpha 2$ globulin (Hackenthal et al., 1990). Ang I is further degraded to the octapeptide Ang II, mainly in the lung, by a zinc-containing dipeptidyl carboxypeptidase, angiotensin-converting enzyme (ACE). Degradation of Ang II by aminopeptidase A (APA) produces angiotensin III (Ang III). Ang II exerts several effects that help to offset the reduced ECFV and the associated decrease in blood pressure (BP). Classically, these Ang II actions include powerful vasoconstriction, increased fluid intake, stimulation of aldosterone release from the adrenal cortex, renal salt retention, and stimulation of the sympathetic nervous system, with several of these Ang II actions mimicked by Ang III (Moeller et al., 1998). Although these classic RAS functions represent an important contribution to physiological regulatory mechanisms, they may, under certain conditions, also turn into pathophysiology inducing arterial hypertension, cardiac and vascular hypertrophy, lung and renal fibrosis, vascular atherosclerosis, or chronic inflammation (Perazella and Setaro, 2003; Marchesi et al., 2008; Forrester et al., 2018). Knowledge of these pathophysiological aspects stimulated the development of inhibitors of the RAS for therapeutic use such as ACE inhibitors, which were introduced into clinical practice 40 years ago with great, ongoing success in combating hypertension and its various sequelae, most notably stroke, or to treat cardiac and renal diseases (Booz and Baker, 1996; Steckelings et al., 2005b; Jones et al., 2008; Lemarié and Schiffrin, 2010).

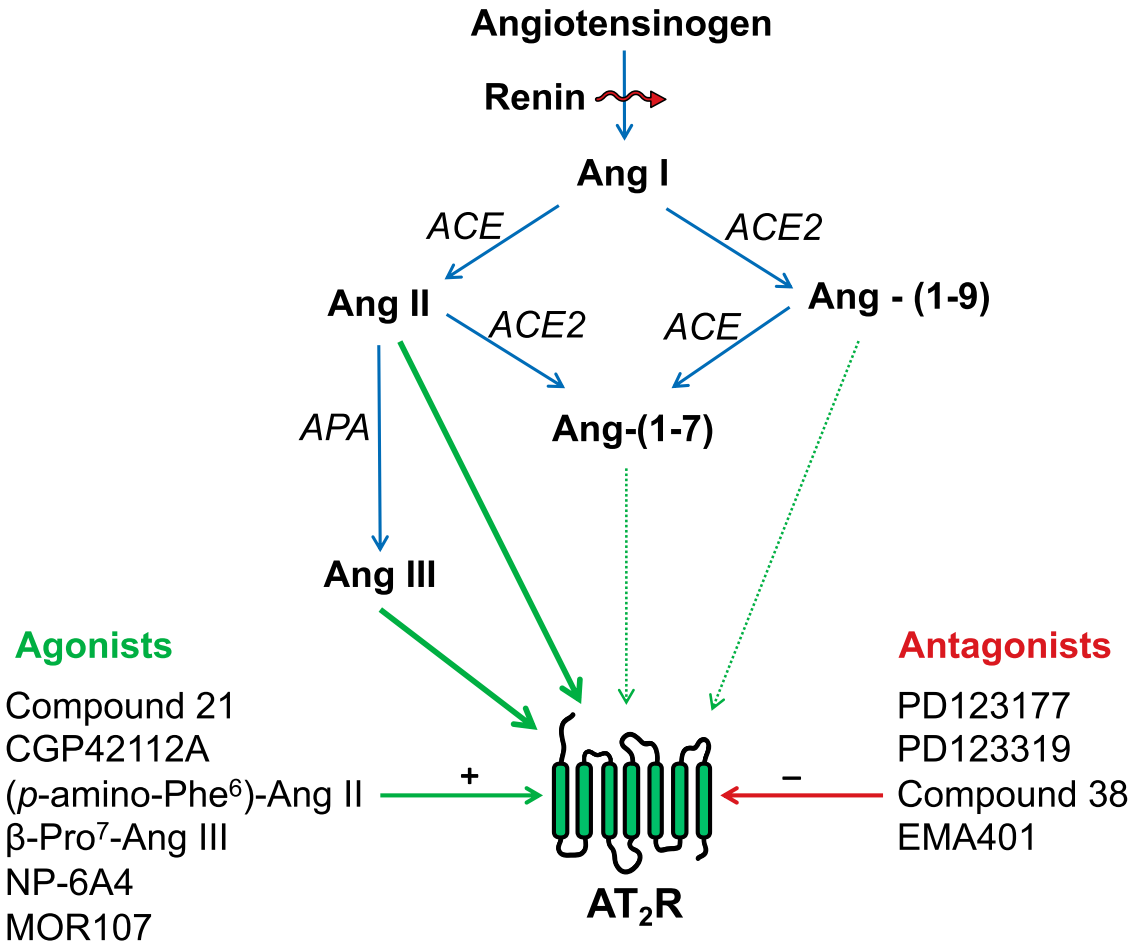

Fig. 1. The angiotensin AT_2 receptor (AT_2R) within the RAS. This diagram depicts the mechanisms of generation of natural (endogenous) peptides that exhibit high (bold green arrows) or lower (dotted green arrows) affinities for the AT_2R and that demonstrate functional activity. Also presented are agonists and antagonists that have been used experimentally to activate/inhibit AT_2R. A detailed discussion of the natural peptides, agonists, and antagonists is provided in Section IV: *AT_2-Receptor Selective Ligands*.

Research during the last decades has added new important new facets to the initial classic RAS concept. First, it was demonstrated that the RAS, with its extension by the adrenal steroid hormone, aldosterone, to the renin-angiotensin-aldosterone system (RAAS), is not only restricted to the circulating blood as a true hormonal system but also exists as the so-called tissue RAS almost ubiquitously in most organs (Lavoie and Sigmund, 2003; Paul et al., 2006). In some organs like the heart, Ang II can be locally generated, but renin may be taken up from the blood for the synthesis of angiotensin peptides (Danser et al., 1994). Second, during the late 1980s, the search for antagonists of the angiotensin receptor was intensified by several groups. This endeavor revealed that Ang II and other peptides of the angiotensin family would bind to least two, if not more, receptor subtypes (Chiu et al., 1989; Whitebread et al., 1989). Although the "classical" effects of Ang II as described above could be attributed to one receptor subtype which was then named the AT_1R, a new receptor subtype with hitherto unknown actions emerged called the angiotensin AT_2 receptor (AT_2R). Ang II, the major effector peptide of the system, was shown to bind with high affinity to both AT_1Rs and AT_2Rs. Losartan (DUP 753), developed by the pharmaceutical company Du Pont, selectively bound to the "classical" AT_1R in blood vessels and other tissues, whereas CGP42112A, PD123177 (EXP-655), and PD123319, developed by Ciba Geigy (Novartis) and Parke Davis, respectively, bound to the "new" angiotensin AT_2R in the uterus and adrenal gland (de Gasparo et al., 2000). Although these compounds were designed to bind selectively to one or the other receptor subtype, only those blocking the known adverse functions of the AT_1R like losartan and the other members of the "Sartan family" were further developed as drugs against hypertension and related diseases (ONTARGET Investigators et al., 2008; Taylor et al., 2011).

Although the AT_2R was initially defined as a high-affinity binding site for Ang II, proof of its existence as a class A GPCR was only revealed with the molecular cloning of the associated cDNA by Victor Dzau's and

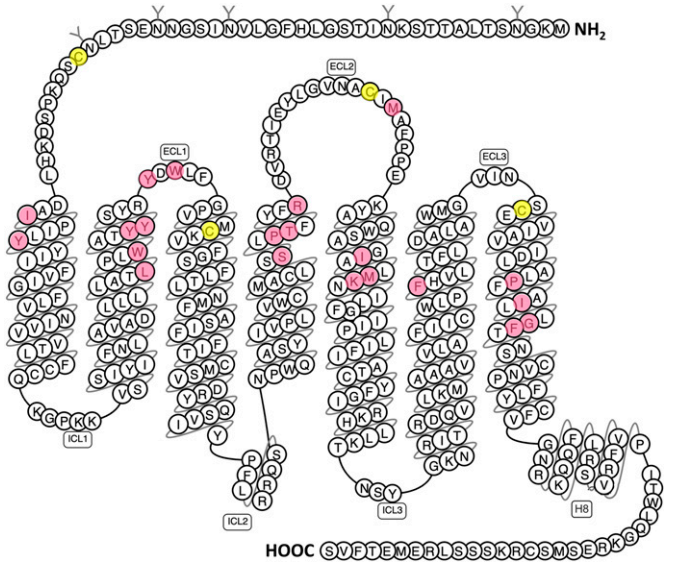

Fig. 2. Snake plot diagram of AT_2R. The cysteines that form disulfides are shown in yellow, important residues for ligand binding are depicted in red, and the glycosylation sites are indicated by "Y."

Tadashi Inagami's groups (Kambayashi et al., 1993; Nakajima et al., 1993). With further characterization of its genomic structure and the documentation of a mouse phenotype by gene deletion experiments by the Dzau-Kobilka and Inagami laboratories (Hein et al., 1995a; Ichiki et al., 1995), the AT_2R was finally recognized as a real biologic entity (Unger, 1999; Gallinat et al., 2000). Nonetheless, it took several years to unveil distinct biologic functions of the AT_2R because of special features of AT_2R activity and regulation. The availability of new pharmacological tools to distinguish between AT_1Rs and AT_2Rs had indicated that these receptors exert completely different and often opposite effects with regard to signal transduction mechanisms and actions on neuronal membrane ionic currents (Pucell et al., 1991; Bottari et al., 1992; Kang et al., 1992, 1993; Mukoyama et al., 1993). The AT_2R was also found to be constitutively active (Miura et al., 2005) so that the regulation of its expression gains importance against the agonist concentrations. It also became apparent that the AT_2R is distinctly regulated. Although it is highly expressed in some fetal tissues, it is usually suppressed in many tissues in the adult, and its expression can be drastically upregulated under the condition of ischemic, inflammatory, or traumatic tissue injury but also in atherosclerotic lesions (Booz and Baker, 1996; de Gasparo et al., 2000; Steckelings et al., 2005b; Jones et al., 2008; Lemarié and Schiffrin, 2010). Thus, investigations into the functional role of the AT_2R were hampered by its low detectability in various organs under "normal" conditions, requiring experimental stimulation of the RAS under selective blockade of the AT_1R to reveal specific AT_2R-mediated actions (Steckelings et al., 2005b). Moreover, vasoconstriction, proliferation, and other features assigned to the AT_1R could not be elicited by AT_2R stimulation, so researchers had to turn to the investigation of novel actions hitherto undescribed with respect to the RAS. The breakthrough regarding its cellular action came with the demonstration of the antiproliferative effect of the AT_2R by two laboratories, using in vitro (Stoll et al., 1995) and in vivo (Nakajima et al., 1995) models. With these studies, it became clear that the AT_2R was not just a weak mediator or effector of classic RAS actions but rather an opponent of the AT_1R within the RAS. This view was fortified by experiments that showed the vasodilating, natriuretic, anti-inflammatory, antifibrotic, even (neuro)-regenerative, and sometimes also apoptotic effects of the AT_2R (de Gasparo et al., 2000; Kaschina et al., 2017).

Over the years, it became increasingly clear that this receptor is part of an endogenous biologic program that can modulate pathological processes induced by AT_1R stimulation or other factors and may enable recovery from disease. This led to a modification of the classic concept of the RAS, where the potentially "harmful" arm of the RAS, represented by the AT_1R, is counterbalanced by the "protective" arm of the RAS. Together with the MAS1 proto-oncogene, G protein-coupled receptor (MAS) receptor, stimulated by the angiotensin peptide fragment angiotensin-(1-7) [Ang-(1-7)] (Paz Ocaranza et al., 2020), the AT_2R has gone through several transformations: from an "enigmatic" receptor to a significant member of the protective arm of the RAS (Unger et al., 2015).

B. Major Functions of AT_2R: The Global Perspective

The AT_2R is one of the main receptors within the protective RAS, others being MAS and insulin-regulated aminopeptidase, and its position within the RAS is illustrated in Fig. 1 along with its endogenous peptide agonists as well as synthetic agonists and antagonists. This "reputation" of the AT_2R (and MAS) is based on a multitude of publications that have documented the protective and regenerative nature of the AT_2R in a broad range of disease models, including cardiovascular, lung, kidney, metabolic, skin, and central nervous system (CNS) diseases as well as in cancer (Mogi et al., 2012; Rodrigues-Ferreira and Nahmias, 2015; Sumners et al., 2015, 2019; Carey, 2017a; Kaschina et al., 2017; Bennion et al., 2018b; Santos et al., 2019; Silva et al., 2020). The therapeutic effect of AT_2R stimulation in these models regularly involves potent anti-inflammatory, antifibrotic, and cell-protective actions (Wang Y et al., 2017; Sumners et al., 2019; Patel et al., 2020). The role of the AT_2R as an endogenous repair and protection system is further supported by the consistent observation from many disease models that in the event of tissue injury, this receptor is upregulated. Moreover, knockdown of the receptor in many models leads to a more severe course

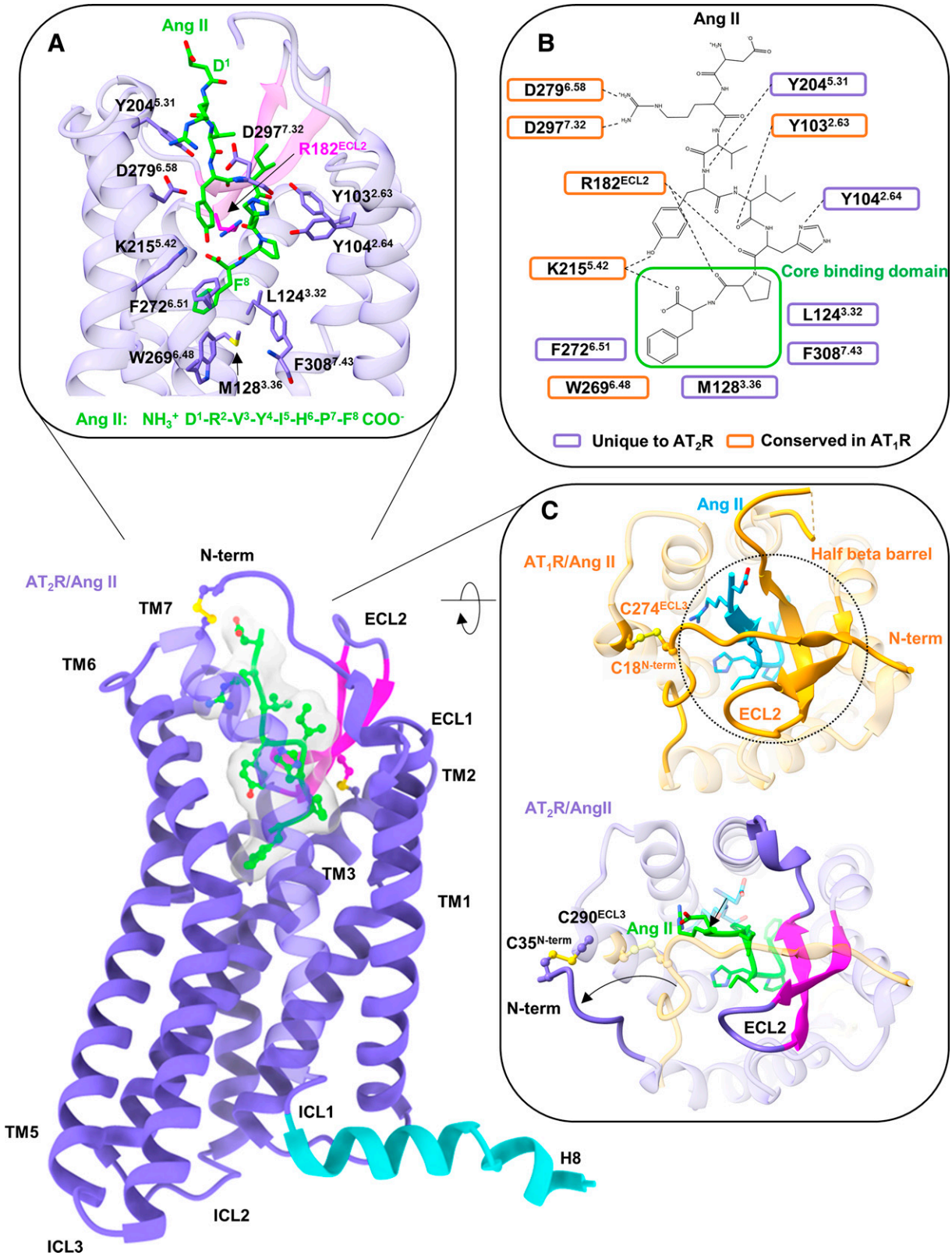

Fig. 3. Overall structure of AT_2R showing the arrangement of transmembrane helices (TM1–7), intracellular loops (ICL1–3), extracellular loops (ECL1–3), amphipathic helix 8 (H8), and the peptide binding pocket (transparent surface around Ang II shown as balls and sticks). (A and B) Interactions with Ang II in the ligand-binding pocket; (C) The peptide backbone of Ang II in the AT_1R but not the AT_2R structure (PDB IDs 6OS0 and 6JOD, respectively) contributes a beta sheet to the ECL2 beta hairpin and the N-terminal beta sheet to form a half beta barrel. The shift in Ang II (cyan sticks and ribbon in AT_1R, green sticks and ribbon in AT_2R) and N-terminal loop positions in AT_2R vs. AT_1R are indicated by arrows. Residue labels in black are of AT_2R.

of disease, indicating that even in the absence of a pharmacological AT_2R agonist, background AT_2R activity (constitutive or by endogenous angiotensin peptides) exerts protective actions.

In contrast to the consistency regarding the protective role of the AT_2R in disease, the role of this receptor in physiological processes is much less well defined. There are several potential reasons why the physiological effects of the AT_2R remain enigmatic. These include: 1) low expression of AT_2Rs in most healthy tissues in contrast to enhanced expression in disease (Booz and Baker, 1996; de Gasparo et al., 2000; Steckelings et al., 2005b; Jones et al., 2008; Lemarié and Schiffrin, 2010); 2) low levels of AT_2R mRNA relative to higher levels of AT_2R protein in tissues such as kidney, similar to the situation with dopamine D_1 receptors (Carey, 2013); 3) the often discrete responses to AT_2R stimulation in the experimental setting (Steckelings et al., 2005b); and 4) the often-inhibitory nature of AT_2R actions that counteract disease mechanisms such as inflammation or fibrosis, which are not present under physiological conditions (Wang Y et al., 2017; Sumners et al., 2019; Patel et al., 2020). Moreover, the reported physiological effects of AT_2R activation involve organ systems that are often very different in their nature from those that are usually associated with the RAS. Examples include an increase in delayed-rectifier K^+ current (I_{Kv}) in neurons (Kang et al., 1992, 1993), the inhibition of cell proliferation (Nakajima et al., 1995; Stoll et al., 1995), or programmed cell death associated with differentiation and development (Horiuchi et al., 1998). More recent research has revealed that there is also a role for the AT_2R within the key domains of RAS function, which are the maintenance of ECFV and BP. In this context, the AT_2R promotes diuresis/natriuresis and lowers BP, the latter including central mechanisms (Sumners et al., 2015; Carey, 2017a).

It needs to be emphasized that the AT_2R is not only a counter-regulator of AT_1R actions (i.e., being dependent on active AT_1R signaling) but that it has its "own life" with an amino acid sequence, an activation mechanism, signaling cascades, and actions in physiology and pathophysiology that are clearly distinct from those of the AT_1R and from most other known 7-TMs/GPCRs.

The difficulty encountered in establishing robust in vitro assays for the detection of AT_2R effects is likely a major reason for the delay in the development of drugs targeting this receptor for therapeutic use of its protective and regenerative properties. Nevertheless, AT_2R agonists (for idiopathic pulmonary fibrosis and COVID-19) and antagonists (for neuropathic pain) have progressed through different stages of preclinical and clinical development, and it can be expected that sooner or later an AT_2R-targeting drug will become available for clinical use.

III. The Angiotensin AT_2-Receptor

A. The AT_2R Gene

1. Location and Structure. The gene that encodes the AT_2R, known as *AGTR2* in humans and *Agtr2* in rodents, is located on the X chromosome (Koike et al., 1994, 1995; Hein et al., 1995b). Traditional radioisotope in situ hybridization studies located the human *AGTR2* to position Xq24-Xq25 and the mouse *Agtr2* to region A2-A4 of the X chromosome (Lazard et al., 1994). Fluorescence in situ hybridization studies assigned the human *AGTR2* to position Xq22-Xq23 (Chassagne et al., 1995) or Xq22 (Tissir et al., 1995) and the rat *Agtr2* to Xq34 (Tissir et al., 1995). The *Agtr2*/*AGTR2* gene exists as a single copy in the genome and consists of three exons: the first two exons encode the 5′ untranslated region, whereas the third exon, which has no introns, contains the entire uninterrupted coding region for the AT_2R (Nakajima et al., 1993; Tsuzuki et al., 1994; Ichiki and Inagami, 1995; Kobayashi et al., 1995). The "intronless" nature of the *Agtr2*/*AGTR2* coding region would indicate that multiple translated forms of the AT_2R, derived by alternative splicing, cannot exist (Grzybowska, 2012; Aviña-Padilla et al., 2021). However, two studies have identified AT_2R mRNA transcripts from heart tissues that exhibited alternative splicing of the 5′ untranslated region of the gene (Wharton et al., 1998; Warnecke et al., 1999), which may potentially influence the rate or efficiency of translation initiation (Kozak, 1991).

Cloning and sequencing of the *AGTR2*/*Agtr2* gene revealed the presence of several *cis*-regulatory DNA elements within the promoter region that can respond to hormones, growth factors, cytokines, or transcription factors and modulate AT_2R transcription (Ichiki et al., 1996; Murasawa et al., 1996). These response elements and factors include activator protein-1 and -2 (AP-1 and AP-2) sites; CCAAT-enhancer–binding proteins (C/EBPs); insulin-response sequence (IRS); interferon regulatory factor (IRF) binding motif; glucocorticoid (GRE); estrogen (ERE)- and cAMP-responsive elements (CREs); nuclear factor 1 (NF-1), NF-IL6, and nuclear factor-kappa B (NF-κB); and poly (ADP-ribose) polymerase-1 (PARP-1) (Horiuchi et al., 1995; Ichiki and Inagami, 1995; Martin and Elton, 1995; Ichiki et al., 1996; Murasawa et al., 1996; Reinemund et al., 2009; Funke-Kaiser et al., 2010; Mishra et al., 2019).

Transcription of the *AGTR2*/*Agtr2* ultimately leads to production of the 363-amino acid AT_2R protein that is highly homologous between humans, rats, and mice. Structural aspects of this class A GPCR are

considered in detail in Section III.B, and the implications of the X chromosome location of *AGTR2*/*Agtr2* for AT_2R biology in females are expanded upon at the start of Section III.E.

2. AGTR2 Gene Mutations. Early studies from two independent groups in which the *Agtr2* was deleted from mice yielded animals that exhibited decreased exploratory behavior or spontaneous movements (Hein et al., 1995a; Ichiki et al., 1995). Subsequently, one of these groups demonstrated that the *Agtr2*-deficient mice also displayed anxiety-like behavior (Okuyama et al., 1999), a phenomenon that is also observed when treating rats with the AT_2R antagonist PD123319 (Moreno-Santos et al., 2021). The conclusions from the studies on *Agtr2*-deficient mice that the AT_2R "played a role in behavior" and the presence of *AGTR2*/*Agtr2* on the X chromosome prompted the investigation of whether the AT_2R is involved in human cognition. The first publication in this regard reported that *AGTR2* mutations may be responsible for altered cognitive function in X-linked mental retardation (XLMR) (Vervoort et al., 2002). Specific findings from this study included: 1) demonstration that a female patient with moderately severe XLMR exhibited disrupted/silenced *AGTR2* transcription due to skewed X inactivation that results in inactivity of both copies of the gene; 2) demonstration of *AGTR2* mutations within eight of 590 male patients with XLMR; these included a frameshift mutation at Phe^{133} that resulted in a truncated AT_2R protein and three different missense mutations that resulted in amino acid substitutions (G21V, R324Q, and I337V) within the extracellular and intracellular domains of the AT_2R. Based on this, the authors concluded that AT_2R has a role in human brain development and cognition (Vervoort et al., 2002). Support for this idea came from a study in which one of the same mutations (leading to G21V) and a novel *AGTR2* mutation (leading to I53F) were identified in a population of male patients with severe nonsyndromic mental retardation (Ylisaukko-oja et al., 2004) and from a study that identified another novel missense mutation of the *AGTR2* (leading to G191E) in a boy with severe mental retardation and pervasive developmental disorder (Takeshita et al., 2012). Further support arose from a preclinical study that demonstrated that AT_2R-null mice exhibit memory deficits and display abnormal dendritic spine morphology and length, characteristics that are present in some mental retardation cases (Maul et al., 2008). However, several other studies challenged whether these identified *AGTR2* mutations exert a causal effect in XLMR. To begin with, sequencing of the entire *AGTR2* coding region in a large population with mental retardation linked to Xq24 revealed no deleterious mutations (Bienvenu et al., 2003). Furthermore, in other studies the frameshift mutations at Phe^{133} and the G21V variant were reported in males from control cohorts, suggesting that they are unlikely to be causative in XLMR (Erdmann et al., 2004; Huang et al., 2005). Further doubt on the causality of *AGTR2* mutations in XLMR was cast by Piton et al. (2013), who used large-scale human exome sequencing via the National Heart, Blood, and Lung Institute (NHLBI) Exome Variant Server (at the time amounting to >10,500 X chromosomes) and classified mutations in the *AGTR2* gene as 'highly questionable' in terms of playing a role in mental retardation with high penetrance in males (Piton et al., 2013). Thus, evidence for a causative role for *AGTR2* mutations in XLMR is currently weak.

Genetic variants of the *AGTR2* gene as a result of single nucleotide polymorphisms (SNPs) have also been described and associated with human disease. These associations include variants associated with: development of congenital anomalies of the kidney and urinary tract (Rigoli et al., 2004; Miranda et al., 2014); gender-specific effects on kidney function and premature arterial aging in type 1 diabetes (Pettersson-Fernholm et al., 2006); hypertension (Jin et al., 2003); hypertension-induced changes in left-ventricular structure (Schmieder et al., 2001); premature coronary artery disease (Alfakih et al., 2005); obesity and body mass index in Japanese women (Kotani et al., 2007); carotid atherosclerosis (Kolaković et al., 2016); and preeclampsia in which the pregnant women had a BMI of >25 (Zhou et al., 2013). AT_2R genotype had no effect on pressor, renal hemodynamic, or aldosterone responses to short-term Ang II infusion (Delles et al., 2000). Individuals carrying the AT_2R 1675G allele may have increased myocardial AT_2R protein expression but not mRNA splicing (Warnecke et al., 2005).

Key Points related to Section III.A on the AT_2R gene are:

- The *AGTR2*/*Agtr2* gene is located on the X chromosome, codes for the AT_2R, which is a class A GPCR, and natural splice variants do not exist.
- *AGTR2* mutations have been associated with XLMR but are unlikely to play a causative role.
- *AGTR2* SNPs have been described in and associated with a variety of human kidney, cardiovascular, and metabolic diseases and with the development of congenital anomalies of the kidney and urinary tract.

B. Structural Determinants for AT_2R Binding and Activation

1. Structure Determination of GPCRs. Despite the involvement of GPCRs in numerous diseases, including cardiovascular disease, structure-based drug design of GPCRs remains challenging due to poor protein expression, low protein stability, intrinsic flexibility leading to several conformational states, and limitations in the

attainable size of crystals for X-ray diffraction experiments (Miyagi et al., 2020). Advances in protein crystallization and data collection have enabled the elucidation of six AT_1R and seven AT_2R crystal structures within the last decade. These structures provide insight into their mechanisms of activation and signaling and also pave the way for structure-based drug design to treat angiotensin receptor (ATR)-related pathologies.

2. Overall Structure of the ATRs. The AT_1R and the AT_2R share 34% amino acid sequence homology, are comprised of 359 and 363 amino acids, respectively, and form part of the class A family of GPCRs. The overall ATR-fold closely resembles that of chemokine and opioid receptors (AT_1R shares 36% sequence identity with C-X-C chemokine receptor 4 (CXCR4) and 33% sequence identity with the κ-opioid receptor) (Zhang et al., 2015). ATRs display the canonical 7-TM alpha helical architecture with a flexible extracellular (EC) N terminus, three intracellular (IC) loops (ICL1–3), three extracellular (EC) loops (ECL1–3), an amphipathic helix 8 (H8), and an IC C terminus (see snake plot diagram of the AT_2R in Fig. 2 and crystal structure in Fig. 3). ECL2 forms a β-hairpin secondary structure, which in AT_1R has been identified as the epitope for a harmful agonistic autoantibody involved in preeclampsia and malignant hypertension (Unal et al., 2012; Xia and Kellems, 2013). The ATR EC side is further shaped by two pairs of disulfides to link the N terminus to ECL3 and to link ECL2 to transmembrane (TM) 3. In most solved GPCR structures, H8 lies parallel to the membrane bilayer regardless of the activation state of the receptor. This orientation, which in some GPCRs is maintained via a membrane anchor to the H8 palmitoylation site, allows for signaling via association with G-proteins and β-arrestin (Escribá et al., 2007). The ATRs lack this palmitoylation site, and a range of H8 orientations are observed in the current ATR crystal structures.

The structures of AT_1R in complex with either its endogenous agonist Ang II (Asp^1-Arg-Val-Tyr-Ile-His-Pro-Phe^8), the partial agonist sarile (Sar^1-Arg-Val-Tyr-Ile-His-Pro-Ile^8) (Sar = sarcosine), or β-arrestin-biased agonists TRV023 (Sar^1-Arg-Val-Tyr-Lys-His-Pro-Ala^8) and TRV026 (Sar^1-Arg-Val-Tyr-Tyr-His-Pro^7) show H8 parallel to the membrane, thus representing the active state of the receptor that is amenable to signaling via G-protein or β-arrestin (Wingler et al., 2019, 2020).

However, the structure of the AT_1R in complex with the small molecule antagonist ZD7155 shows H8 in a noncanonical conformation angled away from the membrane (Fig. 4, A and B), thus representing the inactive state of the receptor, where signaling via G-protein and β-arrestin could be hampered (Zhang et al., 2015).

AT_2Rs do not signal via the canonical Gq/11α/Ca^{2+}/PKC (protein kinase C) stimulation or Giα/oα/adenylyl cyclase inhibition and β-arrestin pathways that are employed by AT_1Rs. It makes sense that AT_2R signaling is different given that AT_2R actions are largely opposing or contrary to those of AT_1R. It is also clear that the AT_2R and AT_1R third intracellular loops (ICL3) are quite different, with the AT_2R ICL3 lacking the consensus motifs required for Gq binding (Mukoyama et al., 1993). Nonetheless, several biochemical (Hayashida et al., 1996; Zhang and Pratt, 1996; Hansen et al., 2000) and functional (Kang et al., 1994, 1995; Hayashida et al., 1996; Horiuchi et al., 1998; Lara et al., 2006; Li J et al., 2007) studies have indicated that AT_2Rs may couple through Gi. However, the first study to investigate the crystal structure of the AT_2R speculated that in the active state, the AT_2R is prevented from associating with G-proteins and β-arrestin (Zhang et al., 2017). Specifically, they demonstrated that binding of the high-affinity small-molecule ligands compound 1 (530-fold selective for AT_2R) or compound 2 (dual AT_1R/AT_2R ligand) to the AT_2R led to a pronounced shift away from the canonical H8 orientation, a configuration that would potentially prevent G-protein and β-arrestin association, and also stabilize the active state of the receptor via extensive hydrophobic and polar interactions with TM3, TM5, and TM6 (Zhang et al., 2017) (Fig. 4B). It is difficult to draw conclusions from this study, as the pharmacological properties of compounds 1 and 2 (whether they are agonists or antagonists) were not revealed and assays to assess G-protein association were not performed. Recently, the crystal structure of the AT_2R complexed to EMA401 was determined (released in the Protein Data Bank (PDB) and accessible via PDB ID 7JNI; there is no associated publication yet) and displayed the same non-canonical H8 orientation as observed with compound 1 or compound 2. EMA401 was derived from the AT_2R antagonist PD123319, but it is not known whether EMA401 acts as an agonist or antagonist (see Sections IV.C.2, IV.C.3, and VI.F.11). Interestingly, in other structural studies it was demonstrated that the crystal structure of the AT_2R complexed to its endogenous ligand Ang II displays the canonical H8 orientation, which could allow for association of these proteins (Asada et al., 2020). Important to note is that the position of H8 was not resolved in the AT_2R structure obtained when using the partial AT_2R agonist sarile as the ligand (Asada et al., 2018; Miyagi et al., 2020). Thus, there remain differing opinions on whether AT_2Rs can interact with and signal via G-proteins. If the AT_2R does interact with Gi, a further consideration that should be made is which intracellular loop of the receptor may interact with the G-protein. Recently, several class A GPCR structures have

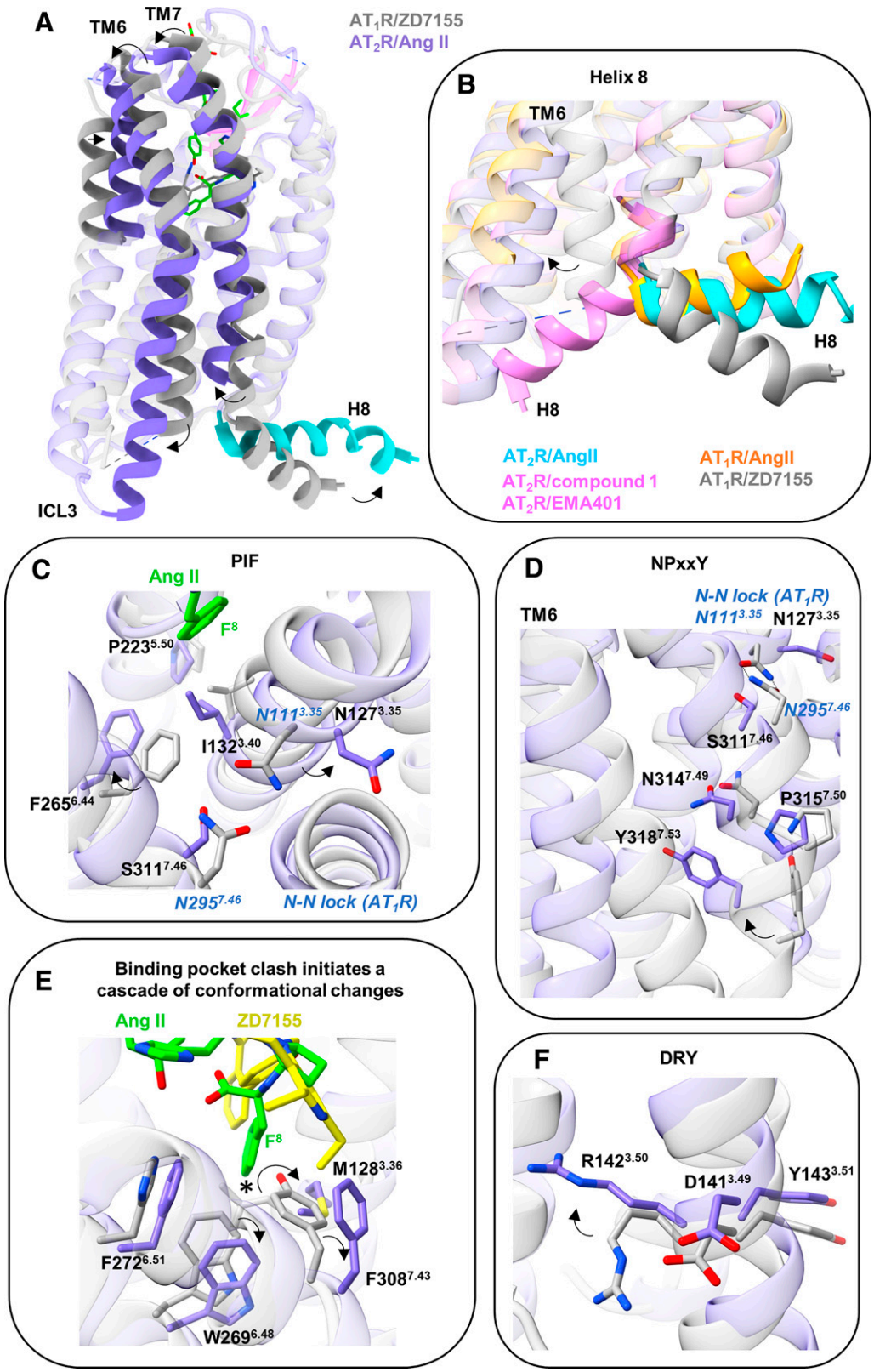

Fig. 4. Comparison between active AT_2R-Ang II (PDB ID 6JOD) and inactive AT_1R-ZD7155 (PDB ID 4YAY) highlights how large-scale conformational changes [indicated by arrows] in (A) TM5-7 and (B) helix 8 (H8) are triggered by rearrangements of the conserved (C) PIF, (D) NPxxY, and (F) DRY motifs, due to (E) steric clashes and rearrangements in the core binding pocket. PDB IDs for AT_1R/Ang II, AT_1R/ZD7155, AT_2R/Ang II, AT_2R/compound 1, and AT_2R/EMA401 are 6OS0, 4YAY, 6JOD, 5UNF, and 7JNI, respectively. Residue labels in black are of AT_2R.

been solved in complex with Gi. These include Gi-complexed serotonin (Huang et al., 2021; Xu et al., 2021), rhodopsin (Kang et al., 2018), μ-opioid (Koehl et al., 2018), and cannabinoid (Xing et al., 2020) receptors in their agonist-bound states, which reveal a common binding site for Gi in an intracellular cleft formed by TM3 and TM7 upon receptor activation. Although functional studies suggest that the AT_2R third intracellular loop is critical for signaling (Kang et al., 1995; Hayashida et al., 1996), it is also apparent that Gi can bind to the second intracellular loop of certain GPCR. Given the proximity between the C terminus of Gi and these loops in class A GPCR-Gi complex structures, both scenarios seem plausible. As the non-canonical conformation of H8 observed in the compound 1/compound 2-AT_2R structures closely interacts with TM3, TM5, and TM6, it is possible that binding of these ligands could prevent G-protein association. Clearly, the topic of G-protein involvement in AT_2R signaling warrants further investigation, as does the influence of the AT_2R/Gi complex on downstream kinase and phosphatase pathways.

3. Peptide Binding to ATRs. The recently determined crystal structures of the AT_1R and the AT_2R in complex with either the endogenous agonist Ang II or the partial agonist sarile (Asada et al., 2018, 2020; Wingler et al., 2019, 2020; Miyagi et al., 2020), with EMA401 (PDB ID 7JNI), with the AT_2R-selective or dual AT_1R/AT_2R ligands compound 1 and compound 2 (Zhang et al., 2017), with the AT_1R inverse agonist olmesartan, or with the small-molecule antagonist ZD7155 (Zhang et al., 2015) allowed comparisons of ligand binding and conserved activation motifs (discussed in Section III.B.4) to reveal the mechanism of ATR activation.

The octapeptide Ang II functions as an endogenous agonist for both AT_1Rs and AT_2Rs by binding to the ligand-binding pocket and interacting with TM2, TM5, TM6, TM7, and ECL2. Sarile, on the other hand, acts as a partial agonist. Small-molecule ligands like compound 1, compound 2, ZD7155, and olmesartan bind to the core binding domain of their receptors, whereas peptides like sarile and Ang II interact with the core as well as the extended binding domain on the extracellular side (Fig. 3, A and B). Structural and mutagenesis data has revealed the importance of core binding domain residues $Arg167^{ECL2}$, $Tyr35^{1.39}$, $Lys199^{5.42}$, and $Trp84^{2.60}$ for both peptide and non-peptide binding to the AT_1R (Zhang et al., 2015).

Peptide binding to both types of receptors occurs in a C-terminal–down conformation with overall very similar receptor conformations between matched pairs (AT_1R/Ang II vs. AT_2R/Ang II or AT_1R/sarile vs. AT_2R/sarile). Although the N-terminal peptide residues (Asp^1/Sar-Arg-Val^3) are in an extended conformation and exposed to solvent at the EC face, the C-terminal residues (Tyr^4-Ile-His-Pro-Phe/Ile^8) are buried and in a C-shaped conformation. This C-shaped conformation contrasts with the S-shaped backbone conformation of the solution structure of Ang II (Spyroulias et al., 2003) and is observed for ATR crystal structures complexed to either Ang II or sarile.

The C-shaped conformation is formed by the constrained dihedral angle of Pro^7, intramolecular interactions between Tyr^4, and the terminal carboxylate of Ile/Phe^8, a collection of hydrophobic interactions to core residues ($Trp100^{2.60}$, $Leu124^{3.32}$, $Met128^{3.36}$, $Phe272^{6.51}$, $Ile304^{7.39}$, and $Phe308^{7.43}$ for the AT_2R), and hydrogen bonding of the peptide backbone to AT_2R $Arg182^{ECL2}$ ($Arg167^{ECL2}$ in the AT_1R) (Fig. 3, A and B), a key residue for small-molecule binding to ATRs (Zhang et al., 2015). The C-terminal carboxylate further forms a salt-bridge interaction with the AT_2R $Lys215^{5.42}$ ($Lys199^{5.42}$ in the AT_1R), which is key for receptor activation, in agreement with mutagenesis data (Noda et al., 1995; Fillion et al., 2013).

A recent review suggested that based on AlphaFold structure predictions (available at https://alphafold.com via UniProt accession codes P30556 and P50052 for AT_1Rs and AT_2Rs, respectively), the N- and C-terminal loops of the two full-length receptors display different conformations (Singh and Karnik, 2022). The predictions for these regions displayed low confidence scores, and the full-length structures have not been experimentally validated since truncated constructs were used for X-ray crystallography. However, some slight changes in sidechain rotamers occur at the peptide N terminus (between matched pairs), which seem to occur because of a difference in the Cys^{ECL3} position, and thus also the receptor N-terminal loop. As reported by Wingler et al. (2019), the AT_1R-sarile structure shows the receptor N terminus wrapped over the EC opening to associate a third β-sheet with the ECL2 β-hairpin, whereas Arg^2-Tyr^4 of sarile contributes a fourth β-sheet, thus forming a half β-barrel. By comparing all ATR crystal structures solved to date, it was observed that this half β-barrel structure occurs in all peptide agonist-bound AT_1R structures but in none of the AT_2R structures (Fig. 3C). In the AT_2R, Asp^1 of Ang II is thus shifted away from TM5 by 3.8Å (relative to AT_1R) along with a change in the sidechain orientations of Asp^1 and Arg^2 (Fig. 3C). Nevertheless, the peptide N terminus is secured in the extended binding pocket of both receptors by key interactions with TM6 and TM7 via $Asp279^{6.58}$ and $Asp297^{7.32}$ in the AT_2R and $Asp263^{6.58}$ and $Asp281^{7.32}$ in the AT_1R (Fig. 3, A and B).

4. Mechanism of ATR Activation. The highly conserved DRY (TM3), NPxxY (TM7), and PIF motifs, which are known to be involved in class A GPCR activation, are conserved in the ATRs (apart from the ionic lock between $R^{3.50}$ of the DRY motif and Asp/

Glu$^{6.30}$ since human ATRs lack an acidic amino acid at position 6.30). Since it is not known whether EMA401 exerts purely antagonistic or partial agonistic effects, there is currently no crystal structure available of inactive AT_2R. Nevertheless, assuming that activation of the two types of ATRs occurs via the same mechanism, insight can be gained into ATR activation by comparing activation motifs (conformational locks) for fully or partially active AT_1R/AT_2R conformations with the inactive AT_1R conformation.

Binding of the C-terminal Ile/Phe^{8} (of sarile or Ang II, respectively) is key for ATR activation by initiating a cascade of conformational changes at the deepest part of the ligand-binding pocket and propagating these signals to the IC side through rearrangements in the activation motifs (Wingler et al., 2019; Asada et al., 2020) (Fig. 4, A–F).

Structures of the active AT_1R (complexed to sarile, biased agonists TRV023/TRV026, or Ang II) are overall very similar but differ from the inactive ZD7155-complexed structure. Changes induced upon agonist binding include a ∼10Å outward displacement of the IC end of TM6, a ∼5Å inward displacement of the IC end of TM7 (where Y302$^{7.53}$ of the NPxxY motif resides), a rotation in H8 to form the canonical conformation parallel to the membrane, an inward rotation of the EC end of TM5, and a slight lateral shift in the EC ends of TM6 and TM7 (Wingler et al., 2019, 2020) (Fig. 4, A and B). Importantly, changes in TM5 and TM6 are linked to rearrangements in the PIF motif (Pro$^{5.50}$-Ile$^{3.40}$-Phe$^{6.44}$) (Fig. 4C) and changes in TM6 and TM7 disrupt hydrogen bonding interactions between Asn111$^{3.35}$ and Asn295$^{7.46}$ (more so if complexed to Ang II vs. sarile or biased agonists) (Wingler et al., 2020) (Fig. 4, C and D). This N-N lock interaction can stabilize the inactive state of AT_1R (Zhang et al., 2015), and agonist-induced rotation of Asn295$^{7.46}$ results in stabilization of the active state via the NPxxY motif. The mechanism of AT_2R activation appears to be similar to that of the AT_1R, with interaction between the AT_2R Met128$^{3.36}$ and Ile/Phe^{8} being key for inducing rearrangements in the activation motifs (Fig. 4E). One difference, however, is the substitution of the AT_1R Asn295$^{7.46}$ with AT_2R Ser311$^{7.46}$ (Fig. 4C). The N-N lock residues form part of the sodium-binding site of class A GPCRs, which is comprised of 16 highly conserved amino acids likely important for receptor activation (Katritch et al., 2014). Interestingly, the Asn295$^{7.46}$Ala mutation led to constitutive activation of the AT_1R, presumably by disrupting the AT_1R Asn111$^{3.35}$ to Asn295$^{7.46}$ hydrogen bonds (Unal et al., 2012). The higher basal activity of AT_2Rs relative to AT_1Rs may thus be explained by the absence of an N-N lock and favoring of the active AT_2R conformation.

Among the class A GPCRs, Ser$^{7.46}$ and Asn$^{3.35}$ are well conserved and allow sodium-mediated allosteric stabilization of TM3 and TM7 in the inactive conformation (Katritch et al., 2014). For AT_1R, sodium was not observed in the antagonist-bound crystal structure, and sodium has a minimal effect on Ang II affinity (Zhang et al., 2015, 2017), meaning that the AT_1R instead relies on the N-N lock to stabilize the inactive state (Wingler et al., 2020). However, since the structure of inactive AT_2Rs has not been solved, it is unclear if the AT_2R (which has Ser311$^{7.46}$) relies on sodium binding to stabilize the inactive state.

In addition, the N-terminal loops and the extent of agonist-induced movement of TM5–7 differ between AT_1Rs and AT_2Rs. The agonist-bound AT_2R structures display a slightly more inward orientation of the EC end of TM6 and a slightly more outward orientation of the EC end of TM7. On the IC side, the end of TM6 is shifted ∼3Å inward in matched pairs of AT_2Rs relative to AT_1Rs. These disparities seem to arise because the N terminus of the AT_1R but not the AT_2R, interacts with ECL2 and the bound agonist by wrapping over the EC groove (Fig. 3C). The Cys$^{\text{N-term}}$-Cys$^{\text{TM7}}$ disulfide bridge in the AT_1R pulls the EC end of TM7 inward due to association between the unique beta sheet in the N terminus of the AT_1R with the ECL2 beta hairpin. Association of peptide agonists with this beta hairpin in the AT_1R results in a shift in Arg^{2} of the peptide, and TM6 is pulled inward via a salt-bridge interaction with Asp263$^{6.58}$ in the AT_1R (Asp279$^{6.58}$ in AT_2R). At present, it is unclear if these differences have any functional significance or if they simply occurred due to the different construct design methods used for AT_1R versus AT_2R crystallization. In contrast to the AT_1R, all AT_2R structures solved to date were of constructs where the N terminus was truncated. The AT_2R/compound 1 or 2 (Zhang et al., 2017) and AT_2R/Ang II (Asada et al., 2020) structures had the apocytochrome b_{562}RIL (BRIL) fused to the N terminus, with the latter also complexed to an AT_2R antibody fragment on the EC side. The AT_2R/sarile structure (Asada et al., 2018) and the latest AT_2R/sarile structure (Miyagi et al., 2020) had BRIL inserted into ICL3, but the former also used an antibody fragment to aid crystallization, whereas the latter had a truncated N terminus. By comparing all ATR structures to date, it appears as though the AT_2R N terminus and ECL2 interactions with the peptide agonists are altered through steric hindrance of BRIL and/or antibody fragments. Given the revolutionary advances in cryo-electron microscopy over the past decade, this technique will likely be useful for the future determination of matched pairs of AT_1R and AT_2R structures in a more native state, potentially even coupled to G-proteins (García-Nafría and Tate, 2019). Regarding H8, the noncanonical orientation observed by Zhang et al. (2017) is unlikely to be a crystallization artifact, as it was observed in different crystal forms and with both compound 1 and 2 ligands. An

important question is, are these compounds (and EMA401 where this noncanonical H8 orientation was also observed) agonists or antagonists of the AT_2R? Although the AT_2R structures complexed to these ligands show the microswitches and transmembrane helices in close agreement with those of other class A GPCRs (including AT_1Rs) in their active states and thus indicate that they may have agonistic properties, the noncanonical H8 conformation suggests that they may be antagonists or partial agonists, as signaling via Gi would most likely be sterically hindered. It is interesting to note that the compound 1/compound 2 ligands extend deeper into the binding pocket of the AT_2R than sarile or Ang II. This is not just true for the ethyl substituent in the R1 position, which causes a shift (relative to the Ang II-AT_2R structure) in the Met128$^{3.36}$ position toward that of the inactive-state Leu112$^{3.36}$ position of AT_1R along with a shift in Phe308$^{7.43}$, but also for the biphenyltetrazole moiety. The structure-activity relationship (SAR) and docking studies performed by Zhang et al. (2017) showed that the biphenyltetrazole moiety of compound 1/compound 2 was reorientated in the AT_1R to align with that of olmesartan/ZD7155 and that a rotamer flip led to a dramatic flip in the quinazolinone moiety compared with the AT_2R. The biphenyltetrazole moiety of compound 1/compound 2 binds deep into a pocket formed by hydrophobic residues of TM3, TM4, and TM5 of the AT_2R, which is not bound in any of the other ATR structures. Another difference between these AT_2R structures and that of Ang II/sarile-bound AT_2R is that Tyr104$^{2.65}$ is flipped out of the binding site by a steric clash with the benzene moiety. EMA401 also induces this rotation in Tyr104$^{2.65}$ but differs from compound 1/compound 2 since it lacks the TM3–5 interactions and displays similar Met128$^{3.36}$ and Phe308$^{7.43}$ conformations as with Ang II. The altered Tyr104$^{2.65}$ conformation caused a steric clash with Leu44$^{N\text{-term}}$ and triggered a change in its orientation along with that of Lys42$^{N\text{-term}}$ and His41$^{N\text{-term}}$, thereby rotating TM1. It is tempting to speculate that this unique clash of Tyr104$^{2.65}$ upon EMA401/compound 1/compound 2 binding could alter the dynamics of TM1, TM2, and the neighboring TM7 to trigger a change in H8 orientation. The inward rotation of the IC side of TM1 upon Tyr104$^{2.65}$ rotation likely causes a clash with the canonical H8 orientation, resulting in its inward motion and association with TM3, TM5, and TM6. As H8 was not resolved in the AT_2R/sarile crystal structures, its orientation is unknown.

Key points related to Section III.B on the structural determinants for AT_2R binding and activation are:

- The ATRs share overall very similar structures in complex with Ang II or sarile.
- Small molecule ligands like compound 1, compound 2, EMA401, ZD7155, and olmesartan bind to the core binding domain of their receptors, whereas peptides like sarile and Ang II interact with the core as well as the extended binding domain on the extracellular side.
- All AT_2R structures to date are in an active-like conformation, including those in complex with EMA401/compound 1/compound 2 where a noncanonical H8 conformation was seen. If these three compounds are antagonists, it suggests that they do not act through conserved microswitches important for AT_1R activation but rather through a unique change in Tyr104$^{2.65}$ to likely prevent G-protein signaling. If they are instead agonists, the change in H8 may trigger biased signaling. To clarify the mechanism of AT_2R activation and signaling, it is crucial to confirm the pharmacological properties of these compounds through functional studies.

C. Intracellular Signaling

AT_2R-induced intracellular signaling is atypical and unlike the traditional modes of signaling displayed by many other GPCRs, including the AT_1R (Kambayashi et al., 1993; Mukoyama et al., 1993). It is also apparent that there is a large diversity of AT_2 R-stimulated signal transduction pathways, with evidence for both G-protein–dependent and –independent mechanisms, which is not uncommon with GPCRs (Hilger et al., 2018; Wootten et al., 2018; Shchepinova et al., 2020). This section will review signaling events that mediate AT_2R-induced cellular actions and functions according to three main phases of signaling. It starts with initial AT_2R signaling (i.e., very early events that require interaction of intracellular proteins with the receptor) followed by intermediate signaling events [i.e., activation of phosphatases, kinases, and peroxisome proliferator-activated receptor-γ (PPARγ)] and finally a brief discussion of downstream signaling pathways that elicit physiological effects or interfere with pathological mechanisms such as inflammation and fibrosis.

1. Initial Signaling – AT_2R-Associated Proteins. There is now much evidence that the association of an inhibitory G-protein (Gi) or AT_2R-interacting protein (ATIP) with the AT_2R is essential for the initiation of signaling by this receptor and that these initial associations lead to subsequent signaling via phosphatase, kinase, and PPARγ pathways (Fig. 5A). Of note, unlike most other GPCRs, the AT_2R does not associate with β-arrestin (Turu et al., 2006). Apart from association with Gi and ATIP, physical interaction of the AT_2R with other receptors (discussed in Section III.D) and with a number of other binding proteins is relevant for the initial steps of signaling, but the

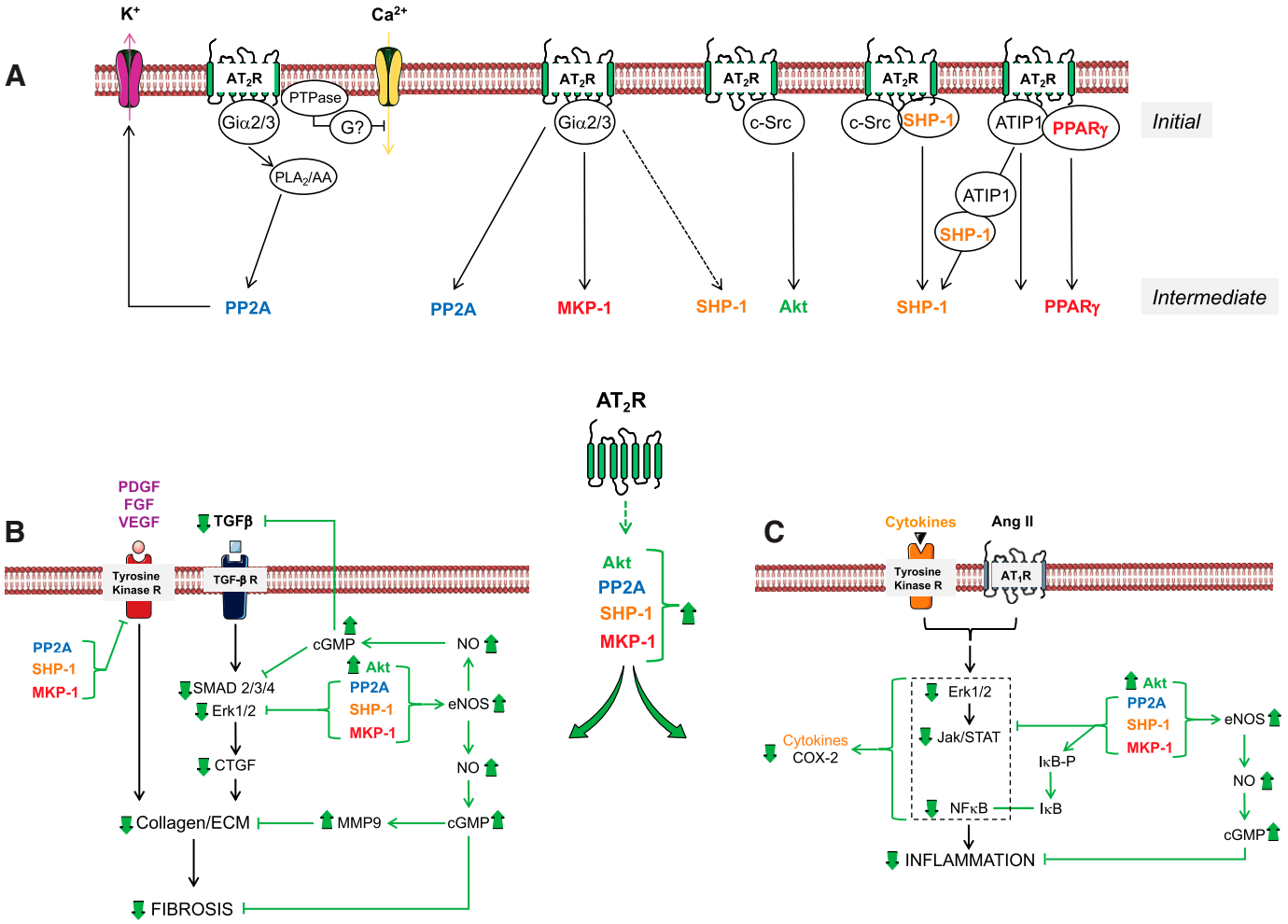

Fig. 5. AT_2R intracellular signaling. (A) Generalized scheme depicting the documented Initial and Intermediate steps in AT_2R-induced signaling. Initial steps include association with proteins such as Gia2/3, ATIP, or c-Src. These associations lead to activation (solid black arrows) of Intermediate signaling molecules; the phosphatases PP2A, MKP-1, and SHP-1; the kinase Akt; and the nuclear protein PPARγ. The dotted black arrow indicates that there is controversy as to the activation of SHP-1 through a G-protein–mediated mechanism. Note that in certain investigations, the role of G-proteins in AT_2R-mediated activation of PP2A or MKP-1 was not studied, so it is conceivable that those phosphatases can also be modulated by a G-protein–independent action. On the left are the signaling pathways that modulate neuronal excitability, and at the center and right are those that modulate downstream signaling events. (B and C) Signaling pathways downstream of PP2A, MKP-1, SHP-1, and Akt that mediate the respective antifibrotic (B) and anti-inflammatory (C) effects of AT_2R stimulation. (B) Black connecting arrows represent the profibrotic actions of tyrosine-kinase and TGF-β receptor activation. Green connecting lines represent the stimulatory or inhibitory actions of AT_2R activation. Solid green up and down arrows represent increases/decreases in molecules or cellular action. (C) Black connecting arrows represent the proinflammatory actions of tyrosine-kinase receptor and AT_1R activation. Green connecting lines represent the stimulatory or inhibitory actions of AT_2R activation. Solid green up and down arrows represent increases/decreases in molecules or cellular action.

impact especially of the latter binding proteins for functional AT_2R-mediated effects is unclear.

a. Signaling via G-protein coupling. Several early radioligand binding studies that demonstrated a lack of ability of guanine nucleotides such as GTP gamma S (GTPγS) or Gpp(NH)p to shift AT_2R-specific binding to a lower affinity state led to the initial conclusion that the AT_2R does *not* signal via G-proteins (Chiu et al., 1989; Dudley et al., 1990, 1991; Bottari et al., 1991; Tsutsumi et al., 1991; Webb et al., 1992; Siemens et al., 1994). In contrast, some studies demonstrated that AT_2R-specific binding, particularly that observed in certain embryonic brain areas and N1E-115 neural cells rich in AT_2Rs, was sensitive to GTPγS (Tsutsumi and Saavedra, 1992; Tsutsumi et al., 1993; Siemens et al., 1994). When the AT_2R was cloned and revealed as a class A GPCR, overexpression of the cloned receptors in COS-7 cells yielded AT_2R-specific binding that was *insensitive* to GTPγS (Kambayashi et al., 1993; Mukoyama et al., 1993), consistent with many of the earlier findings. However, one of the cloning studies also demonstrated that stimulation of the overexpressed AT_2R led to a pertussis toxin (PTX)-sensitive modulation of phosphotyrosine phosphatase (PTPase) activity, implying the involvement of an inhibitory G-protein (Gi or Go) (Kambayashi et al., 1993). They noted that although this was contradictory to the absence of GTPγS-

sensitivity of AT_2R-specific binding, it was not unusual, as similar observations had been made for the dopamine D_3 receptor (Sokoloff et al., 1990), which subsequently was shown to couple via Gi (Robinson and Caron, 1997). The other cloning study noted a structural similarity between the AT_2R and the dopamine D_3 and somatostatin-type 1 receptors (SSTR1s): a conserved 5-amino acid motif (Arg … Leu Lys … … .Arg.Arg) in the ICL3, and suggested that this might be responsible for the lack of traditional signaling of these GPCRs (Mukoyama et al., 1993). Notably, SSTR1s have also displayed variable sensitivity to GTPγS (Rens-Domiano et al., 1992; Buscail et al., 1994; Hou et al., 1994), although they clearly signal via Gi (Hadcock et al., 1994). The conclusions from the cloning studies that AT_2R signaling involved unique or nontraditional G-protein coupling were ultimately supported by a variety of approaches that indicated that AT_2Rs can couple to an inhibitory G-protein.

For example, using diverse cell populations it was demonstrated that cellular and functional effects elicited by AT_2R activation were PTX-sensitive (Kang et al., 1994; Huang et al., 1995, 1996; Ozawa et al., 1996; Yamada et al., 1996; Lehtonen et al., 1999b; Gendron et al., 2002; Li et al., 2004; Moore et al., 2004; Andresen et al., 2005; Li J et al., 2007; Kilian et al., 2008). Although this provided indirect evidence that AT_2R-mediated signaling can involve inhibitory G-proteins, two of the investigations revealed that Gi is likely involved because an AT_2R-mediated increase in neuronal I_{Kv} was inhibited by intracellular perfusion of Giα antibodies (Kang et al., 1994) and transfection of pulmonary artery endothelial cells with a Giα3 dominant negative cDNA blocked the AT_2R-mediated increase in endothelial nitric oxide synthase (eNOS) expression (Li J et al., 2007). These findings were consistent with biochemical studies that demonstrated AT_2R association with Giα2 and Giα3 (Zhang and Pratt, 1996) or with Giα3 alone (Sasamura et al., 2000). Another important finding was the use of radiolabeled GTPγS-binding, which is well suited for studying Gi/o-coupled receptors (Strange, 2010) to demonstrate Ang II- and Ang III-induced activation of Giα via AT_2R in COS-7 cells (Hansen et al., 2000).

The roles of the second and third intracellular loops (ICL2 and ICL3) of GPCR in G-protein signaling are well known (Wong, 2003), with the conserved TM3 DRY (141–143) motif at the N terminus of the ICL2 thought to be essential for G-protein recruitment and signaling (Rosenbaum et al., 2009) (Section III.B; Fig. 4). Mutation of the AT_2R DRY (141–143) motif in CHO-K1 cells resulted in a significant decrease in AT_2 R binding affinity and G-protein recruitment (Moore et al., 2002) as well as reducing the functional ability of the AT_2 R to inhibit insulin-induced extracellular signal-regulated kinase 1/2 (ERK1/2) mitogen-activated protein kinase (MAPK) activation (Moore et al., 2004). Intracellular application of a 22-amino acid peptide corresponding to the ICL3 of the AT_2R-elicited PTX-sensitive actions that mimicked the effects of Ang II via AT_2Rs in two different experimental situations. Namely, an increase in I_{Kv} in cultured neurons (Kang et al., 1995) and also reducing the growth of aortic vascular smooth muscle cells via complexing with Gi and inhibiting ERK1/2 MAPK activity (Hayashida et al., 1996). Further studies from the latter group reinforced the importance of the ICL3 to AT_2R signaling. First, deletion of residues 240–244 or 245–249 within the intermediate portion of the AT_2R ICL3 resulted in either complete or partial loss of AT_2R-mediated inhibition of ERK1/2 MAPK and apoptosis in PC12 cells, respectively (Lehtonen et al., 1999a). Second, similar results were obtained by transfecting PC12 cells with a chimeric AT_1R, the ICL3 of which had been replaced with that of the AT_2R (Daviet et al., 2001). Thus, although G-proteins and in particular Gi are one signaling mechanism used by AT_2R, the first study to report the AT_2R crystal structure raised doubts about this point (Zhang et al., 2017), as discussed in Section III.B. However, the findings of that study are hard to resolve as it is unknown whether the two ligands used in the study (compounds 1 and 2) (Glinka et al., 1994) (US Patent. 5,385,894. 1995) are agonists or antagonists, although compound 1 was developed for use as an AT_2R antagonist (International Patent Application WO95/03055). A more recent investigation that resolved the crystal structure of AT_2R complexed to Ang II demonstrated that it displayed the canonical H8 orientation, which may allow for association of AT_2R and Gi (Asada et al., 2020). In summary, although there is functional, molecular, and biochemical evidence that the AT_2R can use G-proteins as an intracellular signaling mechanism, certain aspects must be clarified, especially concerning the structural approaches.

b. Signaling initiated by AT_2R-interacting proteins. In an attempt to newly identify novel proteins that bind to and interact with the AT_2R, thereby impacting AT_2R signaling and function, two independent groups simultaneously applied yeast-two-hybrid systems and found the same molecule as an intracellular interaction partner with the C-terminal end of the AT_2R, which they termed AT_2R-interacting protein (ATIP; with the human AT_2R 52 C-terminal residues as bait) (Nouet et al., 2004) or AT_2R-binding protein (ATBP; with the mouse AT_2R C-terminal residues 313–363 as bait) (Wruck et al., 2005). Most follow-up studies have been performed on ATIP, the human variant of the AT_2R-binding protein. ATIPs are encoded by the microtubule-associated tumor suppressor 1 (MTUS-1) gene, which was identified as a tumor suppressor gene prior to the discovery of ATIP/ATBP and without any connection to the AT_2R (Seibold et al., 2003). Due

to alternative splicing and alternative promoter usage, transcription of MTUS-1 results in various ATIP isoforms, of which ATIP1 and ATIP3 are the best characterized (Di Benedetto et al., 2006; Rodrigues-Ferreira and Nahmias, 2010). All ATIPs possess a highly conserved C-terminal amino-acid sequence which, in the case of ATIP1, is responsible for binding to the AT_2R. Nevertheless, there is only experimental proof for ATIP1 binding to the AT_2R. Key findings about ATIP1 localization and function from these first two descriptions were 1) ubiquitous expression with the highest abundance in the brain, uterus, and adrenal gland (i.e., tissues in which the AT_2R is also highly expressed); 2) dimerization of ATIPs; 3) coexpression with and binding to the AT_2R but also the ability to exert effects typical for AT_2Rs without binding to the receptor; 4) stronger effects after AT_2R activation and weaker actions in AT_2R-knockout mice (AT_2 R-KO); 5) facilitation of AT_2R trafficking from the Golgi apparatus to the membrane; and 6) antiproliferative effects due to inhibition of receptor tyrosine kinases (Nouet et al., 2004; Wruck et al., 2005). However, although the antiproliferative effect of ATIP1 is generally accepted, the mechanisms by which ATIP1 inhibits receptor tyrosine kinases thus leading to antiproliferation are unresolved. Such mechanisms may involve binding to and activation of Src homology region 2 domain-containing phosphatase-1 (SHP-1) (Li J-M et al., 2007), although others suggested that the antiproliferative effect of AT_2R/ATIP1 is mediated through ATIP1/PPARγ binding and crosstalk (Kukida et al., 2016; Lützen et al., 2017). Recently, research has focused mainly on ATIP3, which seems to be the main isoform possessing tumor-suppressor properties through interference with microtubule depolymerization within the mitotic spindle apparatus (Haykal et al., 2021). It is currently unclear whether the AT_2R plays any role in these anticancer effects of ATIP3 or, for that matter, ATIP1.

The anticancer effects of ATIP3 are reviewed in more detail in Section VI.H and in other reviews (Bozgeyik et al., 2017; Haykal et al., 2021).

c. Other AT_2R-binding proteins. Apart from ATIPs, other AT_2R-binding proteins have been identified by yeast-two-hybrid assays using the C-terminal end of the AT_2R as bait in these hypothesis-generating, "fishing" approaches. In most studies, the interaction of the AT_2R with a binding protein was confirmed by coimmunoprecipitation (co-IP) or colocalization studies, although the relevance of these binding proteins for AT_2R signaling is currently unclear.

Interaction of the transcription factor promyelocytic zinc finger protein (PLZF) with the AT_2R was first described in the context of PLZF-AT_2R cointernalization, PI3-kinase activation, and cardiac hypertrophy, with infusion of Ang II inducing cardiac hypertrophy in wild-type but not in PLZF-KO or AT_2R-KO (Senbonmatsu et al., 2003; Wang et al., 2012). Of note, AT_2R-induced cardiac hypertrophy has only been reported in genetically modified in vitro or in vivo models, whereas pharmacological AT_2R stimulation generally supports an AT_2R-mediated attenuation of cardiac hypertrophy or a neutral effect (see Section VI.B.2) (Steckelings et al., 2010). PLZF also seems to play a role in the neuroprotective effect of the AT_2R (Seidel et al., 2011). Connector enhancer of Ksr (CNK) was shown in human embryonic kidney (HEK)-293 cells transfected with CNK1 and the AT_2R, and also in murine heart tissue, to physically interact at two N-terminal binding sites with the C terminus of the AT_2R (Fritz and Radziwill, 2005). CNK1 is a scaffold protein that plays a role in signaling cascades regulating proliferation, differentiation (including neuronal differentiation), and apoptosis by connecting upstream activators with downstream targets and may connect the AT_2R to Raf/Rho signaling (Jaffe et al., 2004; Ziogas et al., 2005). The tissue inhibitor of metalloproteinase 3 (TIMP-3) was reported to bind to the AT_2R in human ovarian cancer cells resulting in additive inhibition of proliferation and angiogenesis (Kang et al., 2008). Na^+/H^+ exchanger (NHE)-6/AT_2R physical interaction was found using a mouse fetus cDNA library and a human breast cancer cell line (Pulakat et al., 2005), although the functional relevance is not known. ErbB3 is a tyrosine kinase receptor, which is part of the epidermal growth factor (EGF)-receptor family and promotes proliferation and procancerous effects (Kiavue et al., 2020). ErbB3 binding to the AT_2R at the ICL3 and involving the ATP binding site of ErbB3 suggests that ErbB3-AT_2R interaction may prevent transphosphorylation and thus activation of ErbB3, resulting in antiproliferation (Knowle et al., 2000), although experimental proof for this hypothesis is still lacking.

2. Intermediate Signaling.

a. Phosphatases. One of the cornerstones of AT_2R signaling is the activation of protein phosphatases (Fig. 5A), which in many instances leads to interference with kinase-driven signaling pathways (Fig. 5, B and C). This was first indicated with the demonstration of AT_2R-mediated increases in PTPase activity in PC12W cells (Bottari et al., 1992). In the same cell type, it was found that AT_2R activation increased cytosolic PTPase activity and that this was linked to the antigrowth effects of the AT_2R (Inagami et al., 1997). It was also demonstrated that activation of a PTPase was responsible for AT_2R-mediated inhibition of T-type Ca^{2+} current in neuroblastoma cells (Buisson et al., 1995). Many subsequent studies have revealed that the main PTPases that are activated by the AT_2R are MAPK phosphatase 1 (MKP-1) (Yamada et al., 1996; Horiuchi et al., 1997; Fischer et al., 1998;

Guimond and Gallo-Payet, 2012; Wang C et al., 2020) and SHP-1 (Bedecs et al., 1997; Cui et al., 2001; Feng et al., 2002; Shaw et al., 2003; Li J-M et al., 2007; Alvarez et al., 2008; Guimond and Gallo-Payet, 2012). It is also notable that MKP-1 is a dual-specificity phosphatase, exerting serine-threonine phosphatase (PSPase) activity in addition to PTPase activity (Sun et al., 1993). During the same time-period, the serine-threonine protein phosphatase-2A (PP2A) was identified as another AT_2R-coupled phosphatase mediating activation of I_{Kv} in neuronal cultures (Kang et al., 1994; Huang et al., 1995) that was later linked to the neuroprotective action of AT_2Rs (Grammatopoulos et al., 2004). A role of PP2A in AT_2R-induced signaling has since been confirmed by many studies (Moore et al., 2002; Kilian et al., 2008; Bhat et al., 2019; Gildea et al., 2019; Wang C et al., 2020; Kemp et al., 2022). Thus, the main phosphatases that are activated upon AT_2R stimulation are MKP-1, PP2A, and SHP-1.

Current evidence supports two main mechanisms of AT_2R-induced activation of these phosphatases: (i) phosphatase activation by Gi-proteins and (ii) phosphatase activation by phosphorylation, as discussed in the following sections.

(i) Phosphatase activation by Gi-proteins. There is evidence from several studies assessing PTX sensitivity that the coupling of AT_2Rs to the activation of MKP-1 or PP2A is mediated through Gi. For example, AT_2R-induced apoptosis of PC12W cells was dependent on a process that included Gi-dependent activation of MKP-1 (Hayashida et al., 1996; Yamada et al., 1996; Horiuchi et al., 1997). With regard to PP2A, the AT_2 R-induced activation of this enzyme in neuronal cultures was PTX-sensitive (Huang et al., 1995), and the AT_2R-induced activation of neuronal I_{Kv} involved a mechanism that included Gi-induced activation of phospholipase A2 (PLA2), generation of arachidonic acid (AA), and subsequent activation of PP2A (Kang et al., 1994, 1995; Zhu et al., 1998). Similarly, the AT_2 R-induced decrease in ERK1/2 MAPK activity in CHO cells involved a Gi/PP2A-dependent mechanism (Moore et al., 2004). A role of G-proteins in AT_2R-induced activation of SHP-1 was implied by studies in which transfection of the AT_2R ICL3 into PC12 cells activated SHP-1 (Lehtonen et al., 1999a). In another study, AT_2R-induced inactivation of MAP-kinase via SHP-1 in N1E-115 neuroblastoma cells was partially PTX sensitive, though the authors concluded that this event was G-protein independent (Bedecs et al., 1997). Thus, evidence exists that AT_2R-induced activation of PTPases and PSPases is tied to inhibitory G-proteins. A significant gap in the literature is the lack of knowledge of the mechanism by which AT_2R/Gi might activate phosphatases, although clues have been obtained from two studies. Aside from the above-mentioned PLA2/AA/PP2A mechanism in neurons (Zhu et al., 1998), it was shown that stimulation of adult rat ventricular myocytes with CGP42112A-induced carboxymethylation of PP2A by a Gi$\beta\gamma$-PI3-kinase mechanism (Longman et al., 2014). Since carboxymethylation of the PP2A C (catalytic) subunit modulates its assembly into a heterotrimer with the PP2A B and A subunits (Longman et al., 2014), this is a possible mechanism by which AT_2R/Gi/PP2A may elicit signaling.

(ii) Phosphatase activation by phosphorylation. Generally, SHP-1, PP2A, and MKP-1 are known to be activated by phosphorylation at certain amino acid residues. For example, SHP-1 activity is induced through phosphorylation by the tyrosine kinase c-Src (Zhang et al., 2003). This may indeed be the AT_2R-linked activation mechanism of SHP-1 because as shown in fetal rat membrane preparations within 1 minute after AT_2R stimulation, a complex is formed consisting of the AT_2 R, c-Src, and SHP-1, which coincides with SHP-1 phosphorylation, activation, and dephosphorylation of target proteins (Alvarez et al., 2008). AT_2R/c-Src/SHP-1 complex formation was confirmed in rat hindbrain membrane preparations (Seguin et al., 2012). In AT_2R-transfected COS-7 and N1E-115 cells, SHP-1 binding to the AT_2R was evident as well, and AT_2R stimulation resulted in SHP-1 dissociation from the receptor within 5 minutes, coinciding with rapid SHP-1 activation (Feng et al., 2002). Finally, CGP42112A induced phosphorylation of a PTPase in PC12W cells (Abadir et al., 2006); however, the study did not clarify whether the dephosphorylated PTPase was SHP-1 or another PTPase subtype. Interestingly, c-Src also activates Akt (protein kinase B) by phosphorylation at Ser473 (Wong et al., 1999), which is an eNOS activation mechanism used by the AT_2R (Yayama et al., 2006; Peluso et al., 2018).

MKP-1 is reported to be phosphorylated and activated by two different kinases: ERK1/2 MAPK and casein kinase 2α (CK2α) (Slack et al., 2001; Lee et al., 2011). A study investigating early changes in protein phosphorylation in human aortic endothelial cells by time-resolved, quantitative phosphoproteomics provided preliminary evidence that these activation mechanisms may also be part of AT_2R signaling because both CK2α and ERK1/2 MAPK were activated within 1 minute after AT_2R stimulation followed by protein dephosphorylations (i.e., active phosphatases) after 3 minutes (Peluso et al., 2018).

b. Kinases. As discussed above, kinases may be involved in initial AT_2R signaling and link the receptor to phosphatase activation. However, this is still an area that needs experimental confirmation. In contrast, evidence for involvement of kinases, specifically Akt, in intermediate signaling of the AT_2R is strong. Several studies demonstrated AT_2R-induced phosphorylation of Akt at serine 473, which is a known Akt activation

mechanism; others showed an attenuation of AT_2R-induced effects by the Akt inhibitor MK-2206. For example, Akt was identified to mediate the following AT_2R-induced effects: eNOS activation (Hiyoshi et al., 2005; Peluso et al., 2018), improvement of insulin signaling (Quiroga et al., 2018; Dominici et al., 2020), adipose fat browning (Than et al., 2017), proximal tubule albumin endocytosis (Caruso-Neves et al., 2005), and osmotic cellular resistance (Guimarães-Nobre et al., 2021).

c. Peroxisome proliferator-activated receptor-gamma. PPARγ is a nuclear receptor and well known for being critically involved in fat and glucose metabolism. It has been shown by several studies to be involved in AT_2R signaling, but the exact AT_2R-mediated activation mechanism and the positioning of PPARγ in terms of whether it is upstream or downstream within AT_2R signaling are not really clear. Activation may occur by binding to ATIP (Kukida et al., 2016; Lützen et al., 2017), by AT_2R-induced increased PPARγ expression (Zhao et al., 2005; Shum et al., 2013), or by an indirect mechanism such as enhanced binding of nuclear PPARγ coactivators, thereby amplifying ligand-induced PPARγ activity (Zhao et al., 2005). The following AT_2R-mediated therapeutic effects have been related to PPARγ activation: amelioration of insulin resistance in diabetic KK-Ay mice (Ohshima et al., 2012) and obese Wistar rats (Shum et al., 2013), adipocyte differentiation (Shum et al., 2013), attenuation of intima proliferation and vascular inflammation (Kukida et al., 2016), inhibition of vascular calcification in smooth muscle cell-specific AT_2R-overexpressing mice (Kukida et al., 2019), improved outcome after stroke in mice (Shan et al., 2018), and differentiation and apoptosis in human leiomyosarcoma cells in vitro (Lützen et al., 2017). These studies are reviewed from a pathophysiological and therapeutic viewpoint in Section VI.

3. Downstream Signaling. The aforementioned initial and intermediate signaling events (Fig. 5A) link the AT_2R to downstream signaling cascades, which are dependent on the respective cell type and organ as well as the physiological or pathophysiological context. In many cases, AT_2R-induced intermediate signaling interferes with pathological, often kinase-driven downstream pathways, thus attenuating the mechanisms of disease. Since AT_2R downstream signaling mechanisms have been reviewed in detail elsewhere (Rompe et al., 2010b; Guimond and Gallo-Payet, 2012; Namsolleck et al., 2014; Wang Y et al., 2017; Bennion et al., 2018b; Sumners et al., 2019; Patel et al., 2020; Steckelings and Sumners, 2020), we will not focus on the entirety of these pathways in the present review. Rather, we focus on the mechanisms involved in the potent antifibrotic and anti-inflammatory actions of AT_2R stimulation because of the clinical relevance of these areas to AT_2R based therapies (see Sections VI.E and VII).

Illustrated in Fig. 5B are the documented AT_2R-induced signaling events that are downstream of MKP-1, PP2A, SHP-1, and Akt activation and that mediate the antifibrotic effects of AT_2R agonists. Note that this is a generalized scheme designed to cover the mechanisms involved in fibrosis in different organs and tissues such as lung, kidneys, heart, and skin, etc., and all of these events may not occur in each organ or tissue. Central to the action of AT_2R-induced PTPases/PSPases is the inhibition of the profibrotic effects of transforming growth factor-β (TGF-β) and of various growth factors such as platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and vascular endothelial growth factor (VEGF) leading to reduced collagen production and extracellular matrix formation as shown in Fig. 5B and summarized previously (Wang Y et al., 2017; Sumners et al., 2019). The mechanisms by which the AT_2R interferes with TGF-β–induced fibrosis include: 1) dephosphorylation and inactivation of ERK1/2 MAPK with consequent decreased CTGF (connective tissue growth factor) production; 2) activation of eNOS, production of NO, and generation of cGMP, which exerts antifibrotic effects at multiple levels; inhibition of TGF-β production; inhibition of SMAD (Suppressor of mothers against decapentaplegic) phosphorylation and complex formation; generation of MMP-9 (matrix metalloproteinase 9); and direct antifibrotic actions. With regard to growth factor–induced fibrosis, PTPase/PSPase activation elicits inhibition of tyrosine kinase receptor activity by dephosphorylation, thus blunting the effects of growth factors on collagen production and extracellular matrix formation. It is also worth noting that the AT_2R-induced production of NO and generation of cGMP described above are responsible for the vasodilatory action of AT_2R-stimulation (Henrion et al., 2001; Widdop et al., 2003; Batenburg et al., 2004; Peluso et al., 2018).

Illustrated in Fig. 5C are the documented AT_2R-induced signaling events that are downstream of MKP-1, PP2A, SHP-1, and Akt activation and that mediate the anti-inflammatory effects of AT_2R agonists. These events have also been summarized previously (Rompe et al., 2010b; Steckelings and Sumners, 2020). Anti-inflammatory pathways depicted in Fig. 5C are 1) the Akt/PTPase/PSPase-mediated induction of the eNOS/NO pathway leading to cGMP production; 2) inactivation of ERK1/2 MAPK, with consequent decreased Jak/STAT (Janus kinase/signal transducer and activator of transcription protein) activity; and 3) dephosphorylation of I-κB (NF-kappa-B inhibitor), increasing its ability to bind to NF-κB (nuclear factor κB) and thus decreasing the ability of

NF-κB to enter the nucleus and initiate transcription. Collectively, these events lead to reduced synthesis of proinflammatory cytokines and of cyclooxygenase 2 (COX-2), the enzyme that mediates synthesis of proinflammatory prostaglandins.

Key Points related to Section III.C on intracellular signaling are:

- The intracellular signaling pathways coupled to this atypical GPCR are equally atypical and very different from the canonical GPCR-mediated signaling events.
- The initial signaling events modulated by AT_2R-stimulation include both G-protein– and non-G-protein–mediated actions. Activation of protein phosphatases is a core intermediate step in AT_2R signaling, regardless of whether the upstream signaling involves G-proteins or not and is central to activation of the well described anti-inflammatory, antifibrotic, renal, vasodilator, and neuroprotective actions of AT_2R stimulation.
- These signaling pathways afford AT_2Rs the ability to exert protective actions in multiple disease states, sometimes in direct opposition to deleterious AT_1R-mediated effects.

D. Homo- and Heterodimerization

1. AT_2R Homodimerization. Like most GPCRs, the AT_2R undergoes dimerization and forms homodimers and heterodimers with a variety of other receptors (Lyngsø et al., 2009; Farran, 2017).

AT_2R homodimerization was first described by Miura et al. (2005) in PC12W and AT_2R-transfected CHO cells using co-IP and fluorescence resonance energy transfer (FRET). They further reported that AT_2R homodimers constitutively signal resulting in induction of apoptosis. Dimerization and proapoptotic signaling were not altered by AT_2R stimulation, nor did the receptor internalize. Through elegant studies with mutant AT_2Rs, the authors found that AT_2Rs dimerize through a disulfide-bond between Cys35 in the N terminus of one receptor with Cys290 in the third extracellular loop of the other. Homodimerization of AT_2Rs and the involvement of Cys35 were later confirmed in a study by Leonhardt et al. (2017) in HEK-293 cells transfected with AT_2 Rs. Zha et al. (2017) reported AT_2R homodimerization in rat kidney epithelial NRK-52E cells. Homodimerization was boosted by high glucose conditions, which may be a direct effect on the dimerization susceptibility of the receptors or an indirect effect due to increased AT_2R expression under high glucose conditions. Ligand independent homodimerization was also reported by Porello et al. (2011) in transfected HEK-293 cells by protein-fragment complementation assay and GPCR-heteromer identification technology. A single study suggested that in Alzheimer's disease (AD), amyloid β induces the formation of AT_2R oligomers, which are responsible for pathological $G\alpha_{q/11}$-stimulated signaling (AbdAlla et al., 2009). However, this is the only study describing a pathological role of AT_2R homodimers.

2. AT_2R/AT_1R Heterodimerization. AT_2R/AT_1R heterodimerization was first described by AbdAlla et al. (2001) in PC-12 cells, rat fetal fibroblasts, and human myometrial tissue samples using immunoblotting of disuccinimidyl tartarate-stabilized receptor dimers (AbdAlla et al., 2001). Dimerization of the AT_2R with the AT_1R was constitutive and led to inhibition of AT_1 R-mediated G-protein activation and signaling. Attenuation of AT_1R signaling did not require AT_2R activation, as shown by persistence of the effect in cells with dimers containing an AT_2R mutant that is unable to bind agonists or to initiate AT_2R signaling by protein phosphatases. The authors concluded that the AT_2R acts as a kind of inverse agonist of the AT_1R by constitutively preventing conformational changes necessary to initiate AT_1R signaling (AbdAlla et al., 2001). AT_2R-mediated attenuation of AT_1R signaling (calcium signaling; ERK1/2 MAPK activation) as well as constitutive AT_2R/AT_1R dimerization were confirmed in studies by other groups using co-IP, FRET, and bioluminescence resonance energy transfer (BRET) in AT_1 R/AT_2R-transfected HeLa or HEK-293 cells, respectively (Inuzuka et al., 2016; Rivas-Santisteban et al., 2020).

Dimerization of AT_1Rs and AT_2Rs also seems to impact AT_2R intracellular trafficking since $AT_1R/$ AT_2R dimers, but not the AT_2R alone, internalized upon Ang II stimulation and protein kinase C (PKC) activation, which led to phosphorylation of the C-terminal end of the AT_2R (Inuzuka et al., 2016). Internalization of AT_1R/AT_2R/Ang II complexes was also reported by Ferrão et al. (2017) in immortalized pig proximal tubule cells (LLC-PK1 cells) by living cell imaging. The notion that $AT_2R/$ AT_1R dimers are constitutive is challenged by a study in LLC-PK1 cells in which treatment with Ang II increased the occurrence of AT_2R/AT_1R dimers (Ferrão et al., 2012). Treatment with Ang II also led to phospholipase C and sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) activation, which are classic AT_1R signaling pathways. These effects could be blocked with the AT_1R antagonist losartan and the AT_2R antagonist PD123319, the latter presumably through cross-inhibition due to AT_2R/AT_1R dimer formation. Other publications have reported $AT_2R/$ AT_1R heterodimerization in naıve renal tubular epithelial cells (NRK-52E line) (Zha et al., 2017) and in basolateral membranes from proximal tubule cells from sheep (Axelband et al., 2009), Wistar Kyoto (WKY) and spontaneously hypertensive rats (SHRs), where dimers were involved in the regulation of Na^+/K^+-ATPase activity by Ang II and Ang-(3-4) (Dias et al., 2014).

3. AT_2R/MAS Heterodimerization. The AT_2R and MAS are the main receptors of the protective arm of the RAS, and it is striking that their spectrum of signaling mechanisms and actions is almost identical (Villela et al., 2015). Moreover, there are numerous studies in the literature showing that effects of MAS agonists can be inhibited by PD123319 and a few studies showing that effects of AT_2R agonists can be blocked by the MAS antagonist AVE779 (Asp-Arg-Val-Tyr-Ile-His-D-Ala).

Both observations—similarity in signaling and actions and the so-called cross-inhibition—suggest that the AT_2R and MAS dimerize. Experimental proof for this assumption was first provided in 2017 by Leonhardt et al., who provided evidence for AT_2R/MAS heterodimerization by FRET and by cross-correlation spectroscopy in AT_2R-transfected HEK-293 cells (Leonhardt et al., 2017). In primary mouse astrocytes, they further showed cross-inhibition by AT_2R and MAS antagonists and functional interdependence of the AT_2R and MAS since in cells with knockdown of one receptor, the other receptor was nonfunctional (Leonhardt et al., 2017). AT_2R/MAS dimerization intensity was not altered by AT_2R or MAS agonist treatment. Heterodimerization and functional dependence of the AT_2R and MAS were confirmed by Patel et al. (2017) by showing cross-inhibition of AT_2R/MAS effects on natriuresis/diuresis by the respective antagonists in obese Zucker rats in vivo, as well as colocalization and co-IP in kidney sections or cortical homogenates, respectively. Evidence for colocalization and cross-inhibition of the AT_2R and MAS was also found in human umbilical vein endothelial cells by co-IP and colocalization studies (González-Blázquez et al., 2021b).

4. AT_2R/Relaxin Receptor Heterodimerization. Another example of functional interdependence of the AT_2R with a GPCR is the heterodimer formed by the AT_2R and the receptor for relaxin, the relaxin family peptide receptor 1 (RXFP1). As Chow et al. (2014) reported, the antifibrotic effect of relaxin could be blocked by PD123319, and it was absent in an in vivo model of unilateral ureteral obstruction in mice either treated with PD123319 or in AT_2R-KO. Using bioluminescence resonance energy transfer (BRET) in transfected HEK-293 cells, the authors confirmed the existence of constitutive AT_2R/RXFP1 dimers, which were not altered by the respective agonists, Ang II, or H2 relaxin alone or in combination.

A recent study by Chow et al. (2019) added another level of complexity by demonstrating the existence of AT_1R/AT_2R/RXFP1 oligomers in renal and cardiac myofibroblasts, through which, paradoxically and due to cross-inhibition, an AT_1R antagonist inhibited the antifibrotic effects of AT_2R- or RXFP-receptor agonists.

5. AT_2R/Bradykinin Receptor B_2 Heterodimerization. AT_2Rs mediate a vasodilator cascade that includes bradykinin (BK), nitric oxide (NO), and cyclic GMP (cGMP). When AT_2R and BK B_2 receptors (B_2Rs) are activated simultaneously in vivo, NO production is enhanced (Abadir et al., 2003). FRET microscopy and co-IP demonstrated dimerization of AT_2R and B_2R in PC12W cells at baseline in the absence of receptor activation, suggesting constitutive heterodimerization (Abadir et al., 2006). The rate of heterodimer formation was largely a function of the level of AT_2R-B_2R expression. The physical association between the dimerized receptors initiated changes in specific intracellular phosphoprotein signaling activities and enhanced production of NO and cGMP. A combination of AT_2R activation by CGP42112A and B_2R inhibition by icatibant induced maximal heterodimer formation and NO and cGMP formation (by 258% and 80%, respectively) (Abadir et al., 2006). Thus, heterodimerization of the AT_2R and B_2R contributes to the functional enhancement of NO production in PC12W cells, but whether this occurs in other cells is currently unknown.

6. AT_2R/Dopamine Receptor D_1 Heterodimerization. Dimerization of the AT_2R with the dopamine D_1 receptor (D_1R) has been demonstrated in human renal proximal tubule cells by co-IP and FRET microscopy in a single study (Gildea et al., 2012). The selective D_1R agonist fenoldopam increased heterodimer formation on plasma membranes by approximately 2-fold. However, no information is currently available on the regulation or functional significance of AT_2R-D_1R dimerization.

7. AT_2R/Adiponectin Receptor Heterodimerization. One of the most recent findings regarding AT_2R dimerization with other GPCRs is the discovery that AT_2Rs dimerize with adiponectin receptors (AdipoRs) (Zha et al., 2017). Adiponectin is an adipokine, which in obesity and type 2 diabetes is reduced and associated with insulin resistance and early phases of nephropathy. However, in advanced kidney disease, adiponectin levels are elevated and positively predict progression of disease (Choi et al., 2020). Zha et al. (2017) demonstrated that AT_2Rs (and also AT_1Rs) colocalized with AdipoR1 and AdipoR2 in renal tubular NRK-52E cells, but co-IP could only confirm AT_2R/AdipoR2 dimers. The study further identified AT_2R homodimers and AT_1R/AT_2R heterodimers. High glucose increased AT_1R and AT_2R expression and all homo- and heterodimers except for AT_1R/AT_2R dimers, which decreased. Although it seems that dimerization of the AT_1R with adiponectin receptors impaired protective adiponectin effects, understanding the functional role of AT_2R/AdipoR2 dimers requires further investigations.

Key Points related to Section III.D on homo- and heterodimerization are:

- The AT_2R forms homodimers and heterodimerizes with a variety of other GPCRs.
- The functional consequences of AT_2R dimerization are far from being completely understood. However, it seems that the AT_2R, when dimerizing with another atypical, protective GPCR (e.g., MAS), becomes a functional unit with the other receptor, which causes functional interdependence. In contrast, when dimerizing with a classic GPCR such as the AT_1R, which has actions opposing the AT_2R, the AT_2R seems to interfere with signaling of the other receptor in an inhibitory way.

E. AT_2R Expression and Regulation

AT_2R-mediated functional effects in both physiological and pathological situations are diverse (see Sections V and VI), and it follows that AT_2R expression is equally diverse, occurring in a variety of tissues and cells. Although the following sections will detail the tissue expression of AT_2Rs (primarily in rodents and humans), several general features of this expression should first be considered, as well as noting limitations to the methodologies that have been used to determine AT_2R expression.

To begin with AT_2R expression in *healthy adult* humans or animals is, with a few exceptions, generally low (Shanmugam and Sandberg, 1996; de Gasparo et al., 2000), and this likely accounts for the difficulty in identifying physiological actions of this receptor, which is something that has been aided in recent years by the advent of more selective AT_2R agonists (Section IV). In contrast, AT_2R expression within some fetal tissues is extremely high (skin and tongue are notable examples) but declines after birth, becoming low or undetectable in adults (Grady et al., 1991; Feuillan et al., 1993; Shanmugam et al., 1995a). The adrenal is an example for an exception to this general pattern of ontogenetic change in tissue AT_2R expression because AT_2Rs are constantly abundant throughout fetal, neonatal, and adult life (de Gasparo et al., 2000). This general pattern of expression led some to suggest developmental roles of the AT_2R (Akishita et al., 1999; Chamoux et al., 1999; Stoll and Unger, 2001). Another possibility is that this is a system that is established during fetal life and that lays dormant until needed. Indeed, as reviewed previously (Booz and Baker, 1996; Steckelings et al., 2005b; Jones et al., 2008; Lemarié and Schiffrin, 2010) and as described in many of the subsequent sections, tissue AT_2R expression is elevated within diseased adult conditions, presumably helping to exert important protective actions (see Section VI). A further and important feature of AT_2R expression is that it is higher in females versus males in many tissues, including kidney, heart, vasculature, adrenals, CNS, and peripheral nervous system (PNS) (Armando et al., 2002; Silva-Antonialli et al., 2004; Baiardi et al., 2005; Macova et al., 2008; Hilliard et al., 2012; Sampson et al., 2012b). The reasons for this elevated AT_2R expression in females are at least 2-fold. First, the human gene encoding the AT_2R is located on the X chromosome, so normal females carry two copies of the gene versus one in males (Koike et al., 1994). This might suggest greater expression and a greater role for AT_2Rs in females, with the caveat that the extra copy of the AT_2R gene may undergo X-inactivation (Schulz and Heard, 2013). Second, it is established that AT_2Rs are upregulated by estrogen in various tissues (Mancina et al., 1996; Armando et al., 2002; Silva-Antonialli et al., 2004; Suarez et al., 2004; Baiardi et al., 2005; Macova et al., 2008; Hilliard et al., 2013a,b) and that their levels decline postmenopause (Hilliard et al., 2013a). The result of this elevated AT_2R expression in females is that the cardiovascular- and renal-protective effects of AT_2R stimulation are enhanced in females versus males (Sampson et al., 2008; Hilliard et al., 2011, 2012; Brown et al., 2012; Sampson et al., 2012a). In summary, these general factors (young vs. adult; disease; sex) that influence AT_2R expression are considered where appropriate in the following sections.

As will be evident from the following sections, an array of methods has been used to assess AT_2R expression. These range from receptor binding assays (tissue and autoradiography), western blotting, immunofluorescence, reverse-transcription polymerase chain reaction (RT-PCR), in situ hybridization, and AT_2R-GFP transgenic reporter mice. Although each of these methods has provided important information, each has drawbacks. In particular, AT_2R antibody specificity has been a potential problem in AT_2R research as testing of three commercially available antibodies indicated failure to meet rigorous standards for specificity (Hafko et al., 2013), and at least one of those antibodies is no longer available. Nonetheless, other antibodies have clearly demonstrated specificity for the AT_2R (Ozono et al., 1997; Wang et al., 1998; Kemp et al., 2014; da Silva Novaes et al., 2018), fulfilling the criterion that antibodies should not be reactive in cells, tissues, and animals that do not express the target protein. Thus, whether an AT_2R antibody is specific and the results obtained are valid really depends upon the antiserum used, and caution should be observed before making blanket conclusions that AT_2R antibodies are "unspecific." Furthermore, the other techniques that have been used to assess AT_2R have their own limitations. Receptor binding assays using radioligands are useful in detecting the presence of a receptor but lack sensitivity and rely on the specificity of pharmacological agents used as displacers. RT-PCR and in situ hybridization are excellent ways of analyzing the presence of AT_2R mRNA,

but the presence of mRNA does not always mean that protein is present (Greenbaum et al., 2003). AT_2R-GFP transgenic reporter mice allow for visualizing the location of AT_2R containing cells, particularly in the brain, as discussed later in Section III.E.6 (de Kloet et al., 2016b), but these mice cannot be used to determine the exact location of AT_2R within a cell.

1. Kidney. As is the case for the majority of organs and tissues, the renal AT_2R is abundantly expressed during fetal life, but its expression declines within days after birth (Karnik et al., 2015). Early studies showed a high level of AT_2R expression in the embryonic kidney (Gröne et al., 1992). In situ hybridization subsequently demonstrated the first appearance of AT_2R mRNA in the mesonephros and metanephros at embryonic day 12 (E12) and surrounding metanephric S-shaped bodies at E15, but no AT_2R mRNA was observed by in situ hybridization in the kidney after postpartum D22 (Shanmugam et al., 1995b). RT-PCR and radioligand binding autoradiography essentially confirmed this pattern with the AT_2R predominating over AT_1R expression in the kidney throughout fetal life (Norwood et al., 1997; García-Villalba et al., 2003). During embryonic development, AT_2Rs are particularly important for ureteral bud morphogenesis: AT_2Rs are expressed in ureteral bud epithelia during metanephric development, the genetic inactivation or pharmacological antagonism of which leads to impaired ureteral bud branching by downregulation of the glial cell line-derived neurotrophic factor (*GDNF*)/ *REarranged during Transfection (RET) kinase receptor*/ *Wingless*/*Integrated (Wnt)* signaling pathway (Oshima et al., 2001; Song et al., 2010). Deficient ureteral bud branching can induce a substantial reduction in nephron endowment that can lead to renal failure (Sakurai and Nigam, 1998).

Although renal AT_2R expression declines after birth, the mRNA as assessed by RT-PCR is expressed at low levels in the normal adult kidney (Karnik et al., 2015). The AT_2R is typical of several 7-transmembrane G-protein–coupled receptors (e.g., dopamine D_1 and D_5 receptors) that have low levels of mRNA but relatively higher levels of receptor protein expression in the adult (Carey, 2013). Using a specific antibody, Ozono et al. (1997) were first to detect and map the distribution of AT_2R protein in the kidney by immunohistochemistry and western blot analysis. Although AT_2R protein was detected in the E14 rat fetus in undifferentiated mesenchymal cells surrounding epithelial structures and at E19 in S-shaped glomeruli, primitive tubules, and mesenchymal tissue, in young adult (4 weeks) rats, expression was reduced but markedly augmented by sodium restriction (Ozono et al., 1997). Likewise, in mature rats, glomerular and tubule staining was also markedly enhanced by sodium restriction (Ozono et al., 1997). Subsequent studies (Miyata et al., 1999) using RT-PCR and immunohistochemistry confirmed widespread tubular and vascular distribution of AT_2R mRNA and protein in the adult kidney, including proximal tubules, collecting ducts, arcuate arteries, afferent arterioles, and outer medullary vasa recta, with most intense expression in the vasculature of the renal cortex and the proximal tubules of the outer medulla. Compared with these sites, AT_2R expression in the medullary thick ascending limb and glomeruli was relatively low (Miyata et al., 1999). However, AT_2R protein was expressed in human glomerular podocytes as shown by immunocytochemistry and western blot analysis (Liebau et al., 2006). More recent studies have unequivocally demonstrated AT_2R protein in rat renal proximal tubule cells (cytoplasm and apical plasma membranes) by confocal microscopy and electron microscopy immunocytochemistry (Kemp et al., 2014). In the renal vasculature, AT_2 Rs are expressed in both muscular arterioles and also in thin arterioles at much lower levels (Helou et al., 2003). Regarding subcellular distribution, in addition to cytoplasm, AT_2Rs have been localized to nuclear as well as mitochondrial compartments (Gwathmey et al., 2009; Abadir et al., 2011a, 2012). The pattern of renal AT_2R expression, particularly in the proximal tubule, is consistent with its physiological function (see Section V.A).

2. Heart. AT_2Rs are expressed in fetal hearts and in adult hearts of different species [for review, see: de Gasparo et al. (2000), Carey (2005), and Kaschina et al. (2014)]. Similar to other tissues, AT_2 Rs are developmentally regulated with high expression in the neonatal heart and decline after birth (Sechi et al., 1992). In addition, aging and various cardiovascular pathological conditions also upregulate the expression of the AT_2R (Heymes et al., 1998). Autoradiographic studies of the rat heart showed AT_2R expression in the myocardium, in the vascular smooth muscles of the aorta and pulmonary arteries, in the conduction system, and less in the coronary arteries (Sechi et al., 1992). In neonatal and young rat hearts, AT_2Rs were observed in the myocardium and coronary vessels throughout the ventricles (Wang et al., 1998). Using single cell RT-PCR, Busche et al., (2000) demonstrated that in the adult rat about 50% of cardiomyocytes contain the AT_1R, whereas only about 10% carry the AT_2R. After myocardial infarction (MI), expression of AT_2Rs in the rat heart was enhanced and colocalized with inflammatory T cells (Nio et al., 1995; Zhu et al., 2000; Altarche-Xifró et al., 2009; Curato et al., 2010).

In human hearts, AT_2Rs predominate, representing ~75% of angiotensin receptors in normal and noninfarcted myocardium (Wharton et al., 1998) and 50%–80% in ventricles from patients with end-stage heart failure (Regitz-Zagrosek et al., 1996). AT_2R

regulation in heart disease is variable, with studies reporting expression to be increased (Rogg et al., 1996; Tsutsumi et al., 1998), decreased (Regitz-Zagrosek et al., 1995; Matsumoto et al., 2000), or unchanged (Asano et al., 1997; Haywood et al., 1997). However, since these studies determined changes in AT_2R expression in very different pathological entities (ischemic heart disease, valvular dysfunction, heart failure, cardiac fibrosis), differences in expression pattern are probably not surprising. In any case, there is generally an increased AT_2R:AT_1R ratio in human hearts compared with animal hearts (Widdop et al., 2003).

Cellular localization of the AT_2R in the human heart has been examined mainly using emulsion autoradiography (Rogg et al., 1996; Tsutsumi et al., 1998). Most of these studies pointed to its expression in cardiac fibroblasts. For example, in the atrium of patients with coronary heart disease, high densities of the AT_2R were found in fibroblasts at sites of fibrosis (Brink et al., 1996). Similarly, fibroblasts were the major cell type expressing AT_2Rs in the hearts of patients with end-stage ischemic heart disease or dilated cardiomyopathy (Wharton et al., 1998) and in failing hearts (Tsutsumi et al., 1998). In contrast, in patients undergoing coronary artery bypass graft surgery, the AT_2R has been localized in cardiomyocytes (Matsumoto et al., 2000).

Rat coronary endothelial cells (CECs) express both the AT_1R and the AT_2R, as demonstrated by binding studies and by the presence of their respective mRNA through reverse transcription polymerase chain reaction (Stoll et al., 1995). Indirect evidence of the presence of AT_2Rs by functional effects of blockade or activation of this receptor was provided in rat neonatal cardiomyocytes and fibroblasts (van Kesteren et al., 1997), adult rat ventricular myocytes (Fischer et al., 1998), and primary rat cardiac fibroblasts (Lauer et al., 2014).

3. Blood Vessels. AT_2Rs have been localized in vasculature by a range of techniques including mRNA detection, western blot, immunohistochemistry, Ang II-immunofluorescence, radioligand binding, and receptor autoradiography. Vascular AT_2R are developmentally regulated since aorta obtained at embryonic day 18 and postnatal week 2 exhibited predominantly AT_2Rs compared with AT_1Rs, whereas this phenotype was reversed by 8 weeks of postnatal life (Viswanathan et al., 1991). Marked aortic AT_2R expression was also evident in 1-day old rats but not in adult rat vasculature (Nakajima et al., 1995). Generally, AT_2Rs are found throughout the vasculature of adult animals at relatively lower levels than AT_1Rs, although their distribution is not uniform across all species and vessel types and the ontogeny of ATR subtype expression is likely to be vessel specific (Cox et al., 2005). Based on radioligand binding experiments, AT_2Rs were estimated to make up 35%–40% of the ATRs in rat and monkey aorta but were poorly expressed in rabbit aorta (Chang and Lotti, 1991; Viswanathan et al., 1991). AT_2Rs have been detected in a range of rodent resistance vessels such as mesenteric (Matrougui et al., 1999, 2000; Touyz et al., 1999; You et al., 2005), coronary (Akishita et al., 2000b; Wu et al., 2002), and femoral (Akishita et al., 2000b; Wu et al., 2001) arteries; ovine uterine arteries (Burrell and Lumbers, 1997; McMullen et al., 1999); and kidney vasculature (Zhuo et al., 1996; Matsubara, 1998; Miyata et al., 1999), including human renal artery and arcuate and interlobar arteries (Gröne et al., 1992; Goldfarb et al., 1994; Zhuo et al., 1996). Other human vessels reported to express AT_2Rs include coronary (Matsubara, 1998), uterine (Cox et al., 1996), and radial (Zulli et al., 2014) arteries. AT_2Rs are located in endothelial cells and vascular smooth muscle cells in small resistance arteries obtained from rats (Nora et al., 1998; Matrougui et al., 1999; You et al., 2005), and AT2Rs are similarly expressed in primary cultures of arterial and venous endothelial cells (Li et al., 1999; Sohn et al., 2000; Dao et al., 2016; Peluso et al., 2018; Toedebusch et al., 2018).

Compared with male normotensive rodents, AT_2R expression is reported to be greater in the vasculature (and kidney) of females (Silva-Antonialli et al., 2004; Baiardi et al., 2005; Sampson et al., 2008, 2012a; Brown et al., 2012; Dao et al., 2016). Both hypertensive status and aging generally increase vascular AT_2Rs, although there is some variation, particularly in combination (Widdop et al., 2008). In mesenteric vessels from hypertensive rats, levels of AT_2Rs were elevated in young rats relative to normotensives (Touyz et al., 1999) but reduced in adults; the latter phenotype is consistent with reduced *Agtr2* and AT_2 Rs in mesenteric smooth muscle obtained from hypertensive versus normotensive patients (Wen et al., 2019). Interestingly, AT_2R stimulation itself (Abadir et al., 2011b; Toedebusch et al., 2018) or NO (Dao et al., 2016) can increase *Agtr2* and/or AT_2R levels in various cell types including PC12W, endothelial cells, or vascular smooth muscle cells, which occurs in a feed-forward mechanism (Carey, 2017b).

4. Skin. Around the year 1990, a common method of studying angiotensin receptor expression was in situ radioligand binding (receptor autoradiography) in whole body sections of fetal rats. Using this method, the group of Greti Aguilera described the presence of angiotensin receptors in skin as early as 1989 (the year that AT_2Rs were discovered) (Millan et al., 1989). Since the study was performed just before the pharmacological tools to distinguish between receptor subtypes became available, the distribution of AT_1Rs and AT_2Rs in skin remained unresolved. The same group also

described cutaneous angiotensin receptors in the skin of cynomolgus monkey and human fetuses (Zemel et al., 1990). Several follow up studies in the early 1990s used the same technique but in combination with selective AT_1R/AT_2R ligands for displacement of the radioligand to identify the respective receptor subtype. These studies uniformly reported that the AT_2R is the dominating angiotensin receptor in skin from fetal rats (Tsutsumi et al., 1991; Feuillan et al., 1993; Grady and Kalinyak, 1993). It was further reported that the AT_2R is still expressed in skin of adult rats and humans but not at a higher density than the AT_1 R, as occurs in fetal life (Gyurko et al., 1992; Kimura et al., 1992; Viswanathan and Saavedra, 1992; Steckelings et al., 2004; Steckelings et al., 2005a). AT_2Rs were further detected in skin annexes such as eccrine and apocrine sweat glands (Takeda and Kondo, 2001), sebaceous glands (Hao et al., 2011), and hair follicles (Jadhav et al., 2013). Expression of the AT_2R in human skin was recently confirmed by an evaluation of microarray data sets derived from the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/) (Nehme et al., 2015). Studies in primary cutaneous cells allowed identification of the specific cell types expressing angiotensin receptors. In humans, both receptor subtypes were present in keratinocytes, fibroblasts, and dermal microvascular endothelial cells, but in melanocytes only AT_1Rs were found (Steckelings et al., 2004).

Angiotensin receptor expression during skin wound healing is quite well studied and seems to undergo dynamic changes during the three-phasic healing process consisting of 1) hemostasis and inflammation, 2) proliferation, and 3) remodeling and scar formation (Aleksiejczuk et al., 2019; Silva et al., 2020). Details will be discussed in Section VI.D.

Despite strong evidence for AT_1R/AT_2R expression in the skin of various species, which is based on a multitude of studies, general knowledge of the existence of a cutaneous RAS is rather limited, probably because skin is not commonly associated with the RAS, which is still mainly regarded as a hormonal system associated with cardiovascular function.

5. Lungs. Similar to most other organs, AT_2Rs are expressed in the lung during fetal life. As shown by in situ hybridization and receptor autoradiography in rats from 17 days of gestation until day 10 postpartum, receptor expression is most abundant in the bronchial tree but absent in lung parenchyma (Tsutsumi et al., 1991; Shanmugam et al., 1996).

In adult rats and humans, AT_2R expression in healthy lung is low and is often close to the detection limit (Zhao et al., 1996; Chassagne et al., 2000; Parra et al., 2014; Bruce et al., 2015). In humans, AT_2Rs were found in alveolar septae (Parra et al., 2014), in airway epithelial cells of the brush border, in mucous glands, and with lower density in vascular endothelial cells and fibroblasts, as determined by in situ hybridization and immunostaining (Bullock et al., 2001).

Looking at the cellular level in vitro, expression of the AT_2R was reported in rat and human alveolar epithelial cells (AECs) and in primary mouse lung fibroblasts (Wang et al., 1999; Papp et al., 2002; Bechara et al., 2003; Königshoff et al., 2007; Nalbandyan et al., 2018).

Several publications report an increase in AT_2R expression in lung diseases in humans such as pulmonary hypertension (PH) (Chassagne et al., 2000), ethanol intoxication in vitro (Bechara et al., 2003), systemic sclerosis, and idiopathic pulmonary fibrosis as well as in related animal models (Königshoff et al., 2007; Parra et al., 2014; Rathinasabapathy et al., 2018). In contrast, in other diseases (chronic hypoxia, interstitial pneumonia), receptor expression appears unchanged (Zhao et al., 1996; Parra et al., 2014) or even decreased, as in chronic obstructive pulmonary disease (COPD) in humans (Bullock et al., 2001).

6. Nervous System. The view of AT_2R localization in the CNS has evolved considerably. Initial studies used receptor autoradiography or radioligand membrane binding and demonstrated that the level of AT_2R-specific binding is high and very widespread in embryonic and neonatal tissues from rodents, including in the brain (Cook et al., 1991; Grady et al., 1991; Tsutsumi and Saavedra, 1991). These studies also indicated that in *adult* rodent and human brain, AT_2R expression is more limited and localized. In adult rodents, the highest densities of AT_2Rs were shown to be present in areas such as the inferior olive, locus coeruleus, medial amygdala, the medial geniculate nucleus, the superior colliculus, and the tractus olfactorius lateralis (Chang et al., 1990; Gehlert et al., 1991; Obermüller et al., 1991; Rowe et al., 1992; Song et al., 1992; Tsutsumi and Saavedra, 1992), none of which has direct involvement in the well established CNS actions of Ang II to increase water intake and BP. In contrast to rodents, adult (aged) human brain also displayed AT_2R binding in the cerebellum and the substantia nigra (SN) (Barnes et al., 1991, 1993; MacGregor et al., 1995). The location of AT_2Rs in adult rat brain was largely confirmed by mRNA detection via in situ hybridization studies, which also revealed the presence of AT_2R mRNA within the solitary tract nucleus (NTS), cerebellum, and SN (Johren et al., 1995; Lenkei et al., 1996). Within the spinal cord, adult rats contain AT_2R mRNA and protein in the thoracic region, specifically at the intermediolateral cell column (IML) (Ahmad et al., 2003; Chao et al., 2013), a relay point for sympathetic outflow from the CNS. The recent development of an AT_2R-eGFP (enhanced GFP) transgenic reporter mouse and highly sensitive fluorescence in situ hybridization for

the detection of AT_2R mRNA have not only confirmed previous findings but have led to a number of major advances (de Kloet et al., 2016b; Sumners et al., 2020). Namely, the AT_2R-eGFP reporter mouse has revealed a detailed regional pattern of AT_2R-positive cells within adult rodent CNS. This includes their presence in the cerebral cortex, within cardiovascular control centers such as the NTS and rostral ventrolateral medulla (RVLM), and their location adjacent to the paraventricular nucleus of the hypothalamus (PVN), an area that is critical for neurohormonal regulation and sympathetic outflow. These results were supported by fluorescence in situ hybridization (de Kloet et al., 2016b). This transgenic model has also allowed for phenotyping of the specific neuron populations that contain AT_2Rs such as Gamma aminobutyric acid (GABA), glutamate, cholinergic, and catecholaminergic pathways (de Kloet et al., 2016b), which aids in understanding the mechanisms underlying any functional effects of CNS AT_2Rs. As a whole, these studies have revealed that AT_2Rs are present in CNS areas and neuronal pathways that are associated with functional effects of activation of these receptors under normal and disease conditions, as described in Sections V.B and VI.F.

Unlike the CNS, the location of AT_2R in the PNS is a controversial topic, as recently reviewed (Danigo et al., 2021). The first study to indicate an association of AT_2Rs with peripheral nerves demonstrated an upregulation of AT_2R mRNA in the dorsal root ganglia (DRG) and sciatic nerve after transection of the latter (Gallinat et al., 1998). Their conclusion was that the AT_2Rs were most likely associated with Schwann cells rather than the nerve fibers themselves. More recent studies from rats and humans have demonstrated AT_2 on DRG and spinal neurons. Specifically, immunohistochemical studies in rats have shown strong AT_2R-immunopositive staining in nonpeptidergic isolectin B4 (IB4+) C-nociceptor neurons in adult rat DRG (Benitez et al., 2020). The same study also demonstrated that AT_2R immunoreactivity is present, though to a lesser degree, in some peptidergic small C fibers as well as in medium Aδ-neurons and large Aα- and Aβ-neurons (Benitez et al., 2020). AT_2R-immunoreactivity has also been observed in small-diameter DRG neurons and various nerve endings from humans (Anand et al., 2015). The picture of AT_2R location in the PNS is further muddied by a recent study, which demonstrated that these receptors are not localized on DRG neurons derived from mice and humans (Shepherd et al., 2018a). This study included the use of AT_2R-eGFP reporter mice and demonstrated that DRG neurons from these animals lack the AT_2R gene (Shepherd et al., 2018a). Rather, the authors demonstrated the presence of AT_2R on peripheral macrophages and suggest that their activation triggers DRG sensory neuron activation in mice and humans (Shepherd et al., 2018a). In summary, although it is possible that there are species differences in the location of AT_2Rs in the PNS, further studies are required using a technique such as fluorescence in situ hybridization that would enable discrete localization of receptors in peripheral nerves and surrounding cells in both humans and rodents.

7. Pancreas. The AT_2R is expressed in both exocrine and endocrine cells of the pancreas. Early radioligand binding studies demonstrated that pancreatic exocrine acinar AR42J cells express Ang II specific binding sites that are mostly AT_2Rs, with only 10%–15% AT_1Rs (Chappell et al., 1995), and receptor autoradiographic studies revealed that AT_2Rs are expressed throughout the primate pancreas with the highest density present in exocrine acinar cells (Chappell et al., 2001). Localization of AT_2Rs within the exocrine pancreas was further demonstrated by detection of AT_2R mRNA in isolated acinar cells from rat (Tsang et al., 2004).

With regard to the endocrine pancreas, AT_2Rs are expressed in the islets of Langerhans (Lau et al., 2004; Wong et al., 2004; Shao et al., 2013), ductal (Leung et al., 1997b), and stellate cells (Hama et al., 2004). AT_2Rs have been shown to be colocalized with somatostatin containing delta cells in the pancreatic islets (Wong et al., 2004), and other studies have demonstrated that the AT_2R agonist C21 is insulinotropic in pancreatic islets (Shao et al., 2013). Finally, AT_2Rs are expressed in human and rodent fetal pancreatic progenitor cells (Leung et al., 2012), consistent with their role in β-cell development.

8. Adrenal. The localization of AT_2Rs within the adult adrenal gland is well defined. In the rat, radioligand binding, receptor autoradiography, and traditional in situ hybridization approaches have revealed high levels of AT_2Rs within the adrenal medulla (Balla et al., 1991; Heemskerk and Saavedra, 1995; Israel et al., 1995; Lu et al., 1995; Shanmugam et al., 1995a; Belloni et al., 1998; Harada et al., 2010), consistent with its described role at this site in the sympathoadrenal response to various stress stimuli (Belloni et al., 1998; Armando et al., 2004; Saavedra and Armando, 2018). Interestingly, rat adrenal zona glomerulosa also contains small amounts of AT_2Rs (Shanmugam et al., 1995a; Belloni et al., 1998; Macova et al., 2008; Harada et al., 2010; Premer et al., 2013), but these sites have no direct action on aldosterone secretion (Armando et al., 2004; Peters et al., 2012). Consistent with rats, adult human adrenals express AT_2Rs, primarily in the medulla and with low amounts in the zona glomerulosa (Breault et al., 1996; Harada et al., 2010; Vanderriele et al., 2018), although functional effects have yet to be described.

9. Liver. Expression of AT_2Rs in the liver is a controversial topic, with different conclusions reached based on the type of detection method. Based upon mRNA and receptor binding analyses, AT_2R expression in the liver of rodents and humans appears nonexistent or minimal (Bataller et al., 2000, 2003; Paizis et al., 2002; Nabeshima et al., 2006). However, the presence of AT_2R in the liver seems to be supported by findings that carbon tetrachloride (CCl_4)-induced liver fibrosis was greater in AT_2R-KO compared with wild-type mice, but such antifibrotic effects could be secondary as the AT_2R deletion was not liver specific (Nabeshima et al., 2006). Furthermore, and in contrast to the mRNA and receptor binding analyses, studies utilizing immunohistochemistry and western blotting with AT_2R antibodies have suggested the presence of AT_2Rs in rat liver (Wong et al., 2004; Yu et al., 2010). However, as mentioned at the start of Section III, there is controversy surrounding some of the available AT_2R antibodies (Hafko et al., 2013), and one of these studies (Yu et al., 2010) used one of the controversial antibodies. Thus, based on the data that is available so far, it is difficult to conclude whether liver contains AT_2Rs.

10. Intestine. The presence of low levels of AT_2Rs within the ileum, jejunum, and colon of rat intestine was first detected using receptor autoradiography (Sechi et al., 1993) and has been confirmed within the ileum and jejunum using RT-PCR, western blotting, and immunohistochemistry (Ewert et al., 2003b; Wong et al., 2007). In rat colon, a functional assay confirmed the presence of AT_2Rs in the muscular layer that promotes colonic relaxation (Ferreira-Duarte et al., 2021). AT_2Rs are also present within rat duodenal villi, as shown by immunohistochemistry (Johansson et al., 2001). The location of AT_2Rs within human intestine is largely consistent with rats; western blotting and immunohistochemistry demonstrated that they are present in the duodenum (Spak et al., 2019) and jejunum (Casselbrant et al., 2015). AT_2Rs are only weakly expressed in the mucosal layer of the human colon (Hirasawa et al., 2002). The described locations of AT_2Rs within these intestinal regions is consistent with physiological effects on epithelial ion transport and glucose transport, as described in Section V.D.

11. Eyes. In the retinas of young and adult rodents and in humans, AT_2Rs are located in cells and blood vessels within the ganglion cell layer, in the inner nuclear cell layer, in Muller glial cells, and in retinal pigment epithelial cells (RPEs) (Wheeler-Schilling et al., 1999; Sarlos et al., 2003; Sarlos and Wilkinson-Berka, 2005; Kurihara et al., 2006; Senanayake et al., 2007; Downie et al., 2009; Vaajanen et al., 2010; Verma et al., 2019). One study, using immunolabeling, has demonstrated that rats at postnatal day 1 possess AT_2Rs within the hyaloid vessels, a circulatory system that exists transiently and nourishes the developing retina and lens (Sarlos and Wilkinson-Berka, 2005). With regard to nonretinal eye tissues, the presence of AT_2R mRNA has been demonstrated within the choroid and iris/ciliary body of rats (Wheeler-Schilling et al., 1999). The demonstrated locations of AT_2Rs in the eye are consistent with functional effects as reviewed in Section VI.I.

12. Reproductive Organs. *Male:* Ligand binding studies, supported by mRNA measurement, in rat testes revealed that AT_2R expression was highest during early days after birth and decreased with age, and by the fourth week, the receptor expression was very low (Kanehara et al., 1998). The AT_2R expression was mainly localized in interstitial area and seminiferous tubules, as shown by microscopic emulsion autoradiography of testes (Kanehara et al., 1998). Both mouse and human sperm express AT_2R protein and mRNA (Wennemuth et al., 1999; Gianzo et al., 2016). The receptor expression levels in humans correlated well with the sperm motility in fresh semen and prepared sperm cells (Gianzo et al., 2016). Epididymis, particularly the basal region, also expresses AT_2Rs (Leung et al., 1997a).

Female: Human myometrium and uterine leiomyoma express AT_2Rs (ligand binding studies) that are significantly decreased during pregnancy and by oral contraceptives, particularly in myometrium (Matsumoto et al., 2000). Human endometrial tissues also express AT_2Rs (Nakajima et al., 2018). Contrary to the human myometrium and uterine leiomyomas, rat uterine arteries express higher levels of AT_2Rs during pregnancy (Mishra et al., 2018). Several studies have reported that AT_2R proteins and mRNA are expressed in granulosa cells of rat atretic follicles (Daud et al., 1988; Tanaka et al., 1995; Obermüller et al., 1998, 2004; Kotani et al., 1999), and that AT_2Rs are involved in the onset and progression of follicle atresia through induction of granulosa cell apoptosis (Tanaka et al., 1995; Kotani et al., 1999). Other studies performed in rabbits and cows suggest that AT_2Rs are expressed in various parts of the ovary, particularly in follicles (Nielsen et al., 1994; Yoshimura et al., 1996; Schauser et al., 2001).

Key Points related to Section III.E on AT_2R expression and regulation are:

- The introduction of newer, sensitive techniques has revealed that AT_2R expression is more widespread than originally thought, occurring in a range of tissues and cells. Nonetheless, the AT_2R expression level is, with few exceptions, generally low in healthy adults, whereas certain embryonic and neonatal tissues transiently express high levels of AT_2R.

- AT_2R expression is elevated during certain disease conditions, likely contributing to the protective actions of AT_2R agonists.
- Elevated AT_2R expression in females versus males is possibly a reflection of *AGTR2*/*Agtr2* location on the X chromosome and certainly because estrogen upregulates AT_2R expression.

IV. AT_2-Receptor Selective Ligands

A. Peptide Agonists

1. Angiotensin Peptides As AT_2-Receptor Agonists. A number of natural and modified Ang peptides and synthetic ligands (Figs. 6–8) will be discussed, with AT_2R properties of key examples listed in Table 1, some of which are also reviewed elsewhere (Jones et al., 2008; Hallberg et al., 2017; Ranjit et al., 2021).

a. Ang II. The octapeptide Ang II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) exhibits similar nanomolar affinity for both AT_1Rs and AT_2Rs, with a slight selectivity for the AT_2R subtype (Timmermans et al., 1991; Bosnyak et al., 2011; Jones et al., 2011; Del Borgo et al., 2015). However, functional AT_2R effects mediated by Ang II are generally not manifest unless the predominant AT_1R-mediated effects are inhibited (see Sections V and VI).

b. Ang III. Differences between Ang II and Ang III have been extensively documented describing subtle changes in the physiological responses before the discovery of ATR subtypes (Regoli et al., 1974a; de Gasparo et al., 2000). APA-catalyzed removal of the Asp^1 residue in the N-terminal of Ang II provides the heptapeptide Ang III that acts similarly to Ang II on AT_1Rs but is less potent. Ang III exhibits a slightly higher AT_2R:AT_1R affinity ratio than Ang II (Bosnyak et al., 2011; Del Borgo et al., 2015), meaning that the Asp^1 residue is not essential for AT_2R affinity (Regoli et al., 1974b; Bouley et al., 1998; Rosenström et al., 2004a) but that its removal by APA actually enables Ang III to activate AT_2Rs, particularly in the kidney, where it is thought to act as an important endogenous AT_2R agonist (see Section VI.A). Despite the higher affinity of Ang III for the AT_2R, interference with the AT_1R-agonistic properties of Ang III in the brain is used therapeutically by centrally acting APA inhibitors for the treatment of hypertension, which are currently being tested in phase III clinical trials (Llorens-Cortes and Touyz, 2020).

c. Ang-(1-7). Interest in this N-terminal heptapeptide gained significant momentum with the discovery of angiotensin-converting enzyme-2 (ACE2), which catalyzes Ang-(1-7) production from Ang II or Ang I (Turner et al., 2004). Cleavage of the Pro^7-Phe^8 bond in the C-terminal of Ang II by ACE2 has a significant impact on biologic function and delivers the heptapeptide Ang-(1-7), which is part of the protective RAS and counteracts AT_1R functions (Santos et al., 2000; Santos and Ferreira, 2007). Ang-(1-7) is generally considered to signal via the receptor MAS (Santos et al., 2003, 2006), although it may also act as a low-affinity AT_2R agonist. For example, Ang-(1-7) exhibits a 40-fold selectivity for AT_2Rs over AT_1Rs in radioligand binding studies, although its AT_2R affinity is ~500-fold less than that of Ang II (IC_{50} value ~0.2 μM) (Bosnyak et al., 2011). Ang-(1-7) effects could be blocked by the AT_2R antagonist PD123319 (De Souza et al., 2004; Castro et al., 2005; Walters et al., 2005; Lara et al., 2006; Bosnyak et al., 2012). However, PD123319 is not only an antagonist for the AT_2R but also for the MAS-related G-protein–coupled receptor D (MrgD), the receptor for alamandine, which is derived from Ang-(1-7) by decarboxylation (Lautner et al., 2013).

d. Ang-(1-9). The nonapeptide angiotensin-(1-9) [Ang-(1-9)], containing a histidine residue at the C-terminal, is formed from Angiotensin I after hydrolysis of the carboxy terminal leucine by ACE2 and was generally not considered to be biologically active. However, Ang-(1-9) evoked antihypertrophic and antifibrotic effects in cardiac tissue in vitro and in vivo, in part via AT_2R activation (Ocaranza et al., 2010, 2014; Flores-Muñoz et al., 2011; Flores-Munoz et al., 2012), although Ang-(1-9) binds with only moderate affinity at the AT_2R, which was >100-fold lower than Ang II at AT_2R (Flores-Muñoz et al., 2011). Ang-(1-9) is reported to inhibit cardiac ACE and to potentiate BK signaling at concentrations that are at least 10-fold lower than its pKi for AT_2R (Erdös et al., 2002), which may contribute to the effects reported. Local gene delivery of Ang-(1-9) to the heart protected against MI-induced injury (Fattah et al., 2016), although the ATR subtype involvement was not investigated.

e. Ang IV. Cleavage of the Arg^2-Val^3 bond provides the hexapeptide angiotensin IV (Ang IV) with a 100-fold lower AT_2R affinity than Ang II and Ang III, demonstrating the importance of the Arg^2 residue for binding of Ang II analogs to the receptor. Interestingly, Ang IV exhibited ~5-fold greater AT_2R:AT_1R selectivity than Ang-(1-7) (Bosnyak et al., 2011). Ang IV exhibits a very different pharmacological profile than Ang II (Harding et al., 1994; Wright et al., 1996) and acts as a cognitive enhancer in experimental models (Braszko et al., 1988), a finding that prompted the search for Ang IV peptidomimetics as a new class of potential pharmaceutical agents for treatment of cognitive disorders (Albiston et al., 2008; Hallberg, 2009; Hallberg and Larhed, 2020). The effects of Ang IV are complex, as it is also an endogenous inhibitor of the enzyme insulin-regulated aminopeptidase (Albiston et al., 2001, 2008) and exerts vasoprotective effects that were inhibited by AT_2R blockade (Vinh et al., 2008a,b).

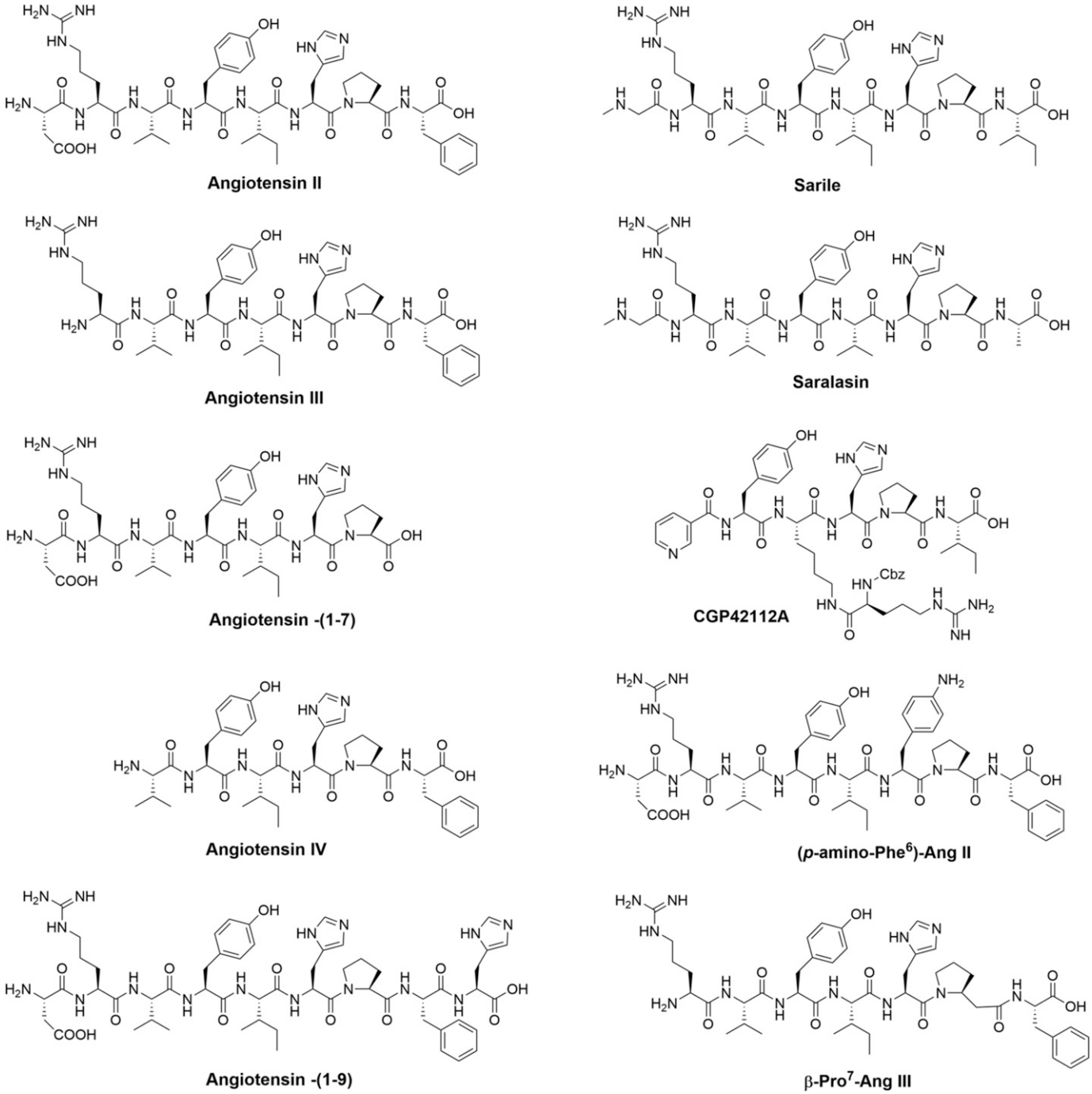

Fig. 6. Five endogenous angiotensin peptides (left) and five modified angiotensin peptides (right) that are useful research tools.

2. Synthetic Peptides Acting on the AT_2R.

a. Sar^1-Ang II, Sar^1 Ile^8-Ang II, and Sar^1 Val^5 Ala^8-Ang II. The octapeptides sarile (Sar^1-Ile^8-Ang II) and saralasin (Sar^1-Val^5-Ala^8-Ang II) were used as Ang II receptor blockers in clinical studies several decades ago (Pals et al., 1971; Hata et al., 1978), prior to the introduction of the ACE inhibitors (Ondetti et al., 1977). Both peptides encompass a sarcosine (Sar) amino acid residue in the N-terminal, whereas the Phe^8 in the C-terminal of Ang II is replaced with Ile^8 in sarile and Ala^8 in saralasin. (Fig. 6) The two octapeptides bind both to AT_1Rs and AT_2 Rs with approximately the same affinity (Criscione et al., 1990). The C-terminal determines the functional response when binding to AT_1Rs (i.e., AT_1R agonism with a C-terminal Phe^8 and AT_1R antagonism or partial agonism with an aliphatic side chain, as in the case of sarile and saralasin) (Aumelas et al., 1985). Recently, it was reported that sarile and saralasin act as AT_2R agonists

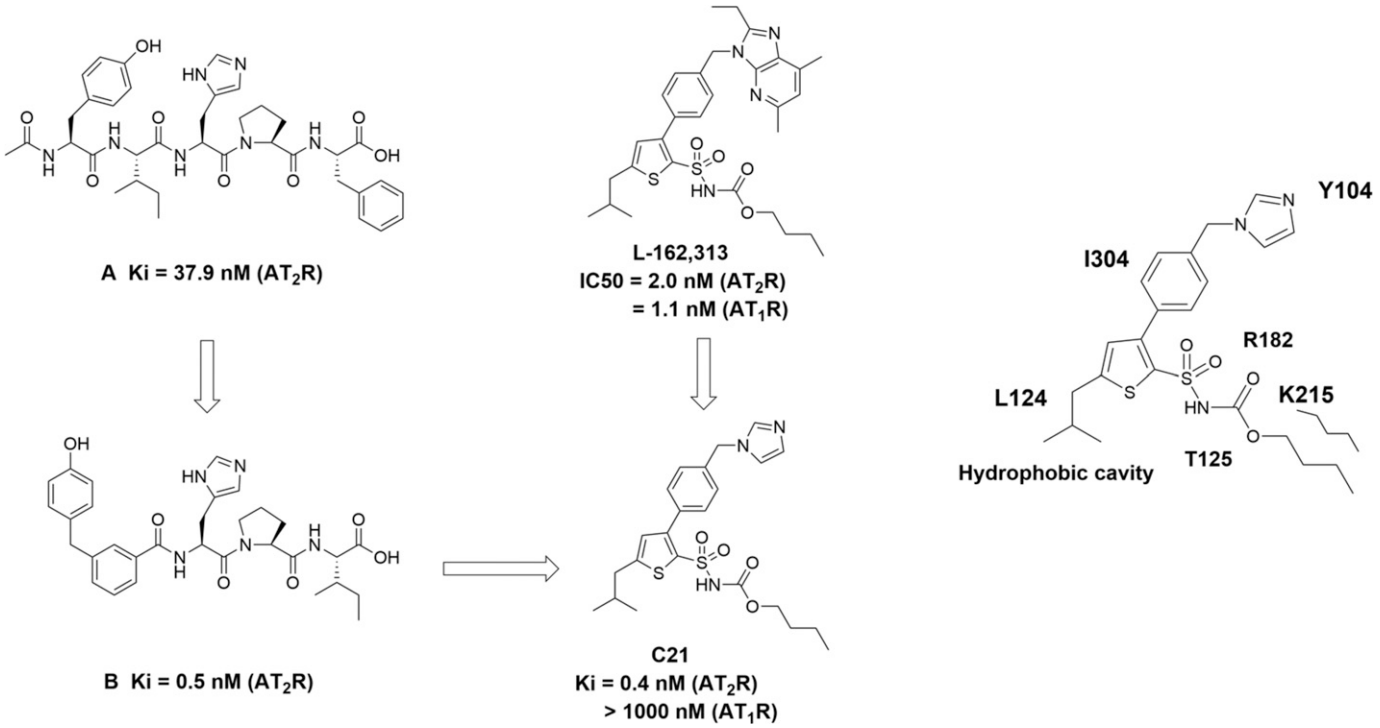

Fig. 7. Structural similarities of the short peptides A and B with C21, all acting as AT_2R-selective agonists are shown. The nonselective thiophene derivative L-162,313 was the starting point for the synthesis of C21. Proposed molecular interactions when C21 binds to AT_2R are depicted on the right-hand side of the figure. As deduced from modeling and molecular dynamic simulations, C21 is engaged in electrostatic interactions with the positively charged $K215^{5.42}$ and $R182^{4.64}$ of AT_2R, and a hydrogen bond between NH of the sulfonyl carbamate and $T125^{3.33}$ is created. The aliphatic chain of the butyloxycarbonyl group is extending parallel to the $K215^{5.42}$ sidechain, and the isobutyl group of C21 is occupying a hydrophobic cavity formed by residues from TM2, TM3, TM6, and TM7. The imidazole ring forms a hydrogen bond with $Y104^{2.65}$ and thereby provides a second electrostatic anchoring point of importance.

and induce neurite outgrowth in NG108-15 cells (Guimond et al., 2014). Hence, it is tempting to speculate that AT_2R activation rather than AT_1R blockade might account for some of the previously reported data obtained with sarile and saralasin as ligands.

b. CGP 42112A. The AT_2R selective peptide ligand CGP42112A (AT_1R; inhibition constant [K_i] = 568 nM and AT_2R; K_i = 0.73 nM) has been used as a valuable research tool and prototype AT_2R agonist for many years (Whitebread et al., 1989) (Fig. 6). The C-terminal of CGP42112A comprises His-Pro-Ile, as in the C-terminal of the nonselective peptide sarile. The nicotinyl amide function in the N-terminal of CGP42112A underscores that the Asp^1 residue is not important for binding affinity to the AT_2R (Yee et al., 1998; Hines et al., 2001), which fits with more recent studies using Ang III analogs (Del Borgo et al., 2015). Interestingly, it is notable that CGP42112A is lacking the Arg^2 residue in the N-terminal that is considered crucial for AT_2R affinity in linear Ang II analogs (Heerding et al., 1997; Knowle et al., 2001; Sköld et al., 2008), based on Ala (Miura and Karnik, 1999) and Glu (Rosenström et al., 2004a) scans. A large series of Ang analogs comprising a variety of turn mimetics have been synthesized and examined in efforts to determine bioactive conformations at AT_2R (Rosenström et al., 2004b; Georgsson et al., 2005; Rosenström et al., 2006).

c. Position 6 substitutions. The octapeptide (*p*-amino-Phe^6)-Ang II (AT_2R; K_i = 0.7 nM) provides a second example of a selective AT_2R agonist applied as a research tool, although it is less commonly used than CGP42112A (Speth and Kim, 1990). In the C-terminal of (*p*-amino-Phe^6)-Ang II, a 4-amino-Phe-Pro-Phe tripeptide fragment replaces the His^6-Pro^7-Phe^8 residues of Ang II (Fig. 6). Additionally, replacement of the histidine residue in position 6 of Ang II for a tyrosine also resulted in a highly selective AT_2R ligand ([Y]^6-Ang II) deduced from binding studies (Magnani et al., 2014), which, together with triazole-Tyr^6-Ang II analogs, exhibited agonist activity since they promoted neurite outgrowth (Vrettos et al., 2020) and inhibited MCF-7 breast carcinoma cellular proliferation (Magnani et al., 2014).

d. Beta-amino acid substituted Ang peptides. Another strategy used to develop AT_2R-selective peptides has been to incorporate β-amino acid substitutions in the peptide backbone. Initial β-amino acid scans using Ang II as a template resulted in ligands with high AT_2 R selectivity (~1000-fold greater than for the AT_1R based on radioligand binding studies), exemplified by methylene group extended peptides β-Tyr^4-Ang II and β-Ile^5-Ang II (Jones et al., 2011). These peptides behaved as agonists and evoked vasodilator responses in vitro and in vivo. Given the modest AT_2R selectivity

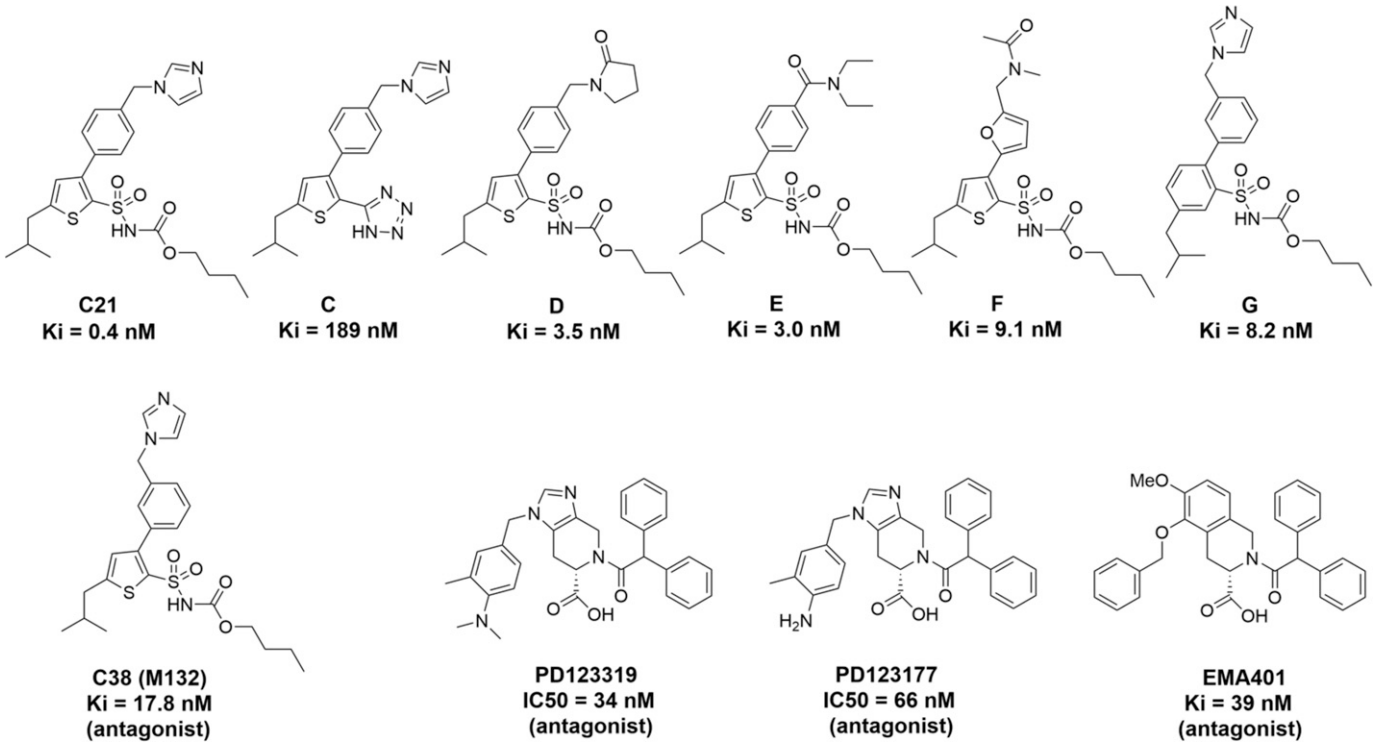

Fig. 8. The AT_2R-selective agonists C21, C, D, E, F, and G that induce neurite outgrowth in NG108-15 cell assays and the AT_2R selective ligands C38 (M132), PD123319, PD123177, and EMA401 that are reported to act as antagonists at AT_2R.

of Ang III over Ang II (Bosnyak et al., 2011), analogous β-amino acid scans were performed using an Ang III template culminating in β-Pro^7-Ang III (Fig. 6). This peptide exhibited a >20,000-fold AT_2R:AT_1R selectivity and evoked vasorelaxation and marked vasodepressor effects in conscious SHR against a background of AT_1R blockade that was sensitive to blockade by PD123319, indicative of AT_2R agonism (Del Borgo et al., 2015). More recently, β-Pro^7-Ang III evoked renal vasodilation and caused natriuresis similarly to Ang III in normotensive rats during AT_1R blockade (Krause et al., 2020), highlighting it as a useful selective tool to interrogate AT_2R function.

e. Other synthetic peptides as AT_2R agonists. The lanthipeptide LP2/3 [dKcAng-(1-7)], also known as MOR107, is a cyclic peptide that resists enzymatic degradation through an introduction of a D-lysine in the N terminus. No published data are available on the relative AT_2R selectivity of this compound, which evoked protective effects in an hyperoxic lung injury model (Wagenaar et al., 2013, 2014). A pharmacokinetic and safety phase I study demonstrated that LP2/3 was relatively stable, with a terminal half-life of ~2 hours in plasma (Namsolleck et al., 2021). The hexapeptide novokinin (Arg-Pro-Leu-Lys-Pro-Trp) exerted low micromolar affinity in AT_2R-transfected Hela cells and evoked vasorelaxation that was blocked by PD123319 (Yamada et al., 2008a,b). Another hexapeptide, NP-6A4 (Lys-4Hyp-Leu-Lys-Pro-Trp), containing a 4-hydroxyproline residue, has been reported to be a selective AT_2R peptide agonist and exerted beneficial effects in models of cardiometabolic disease (Mahmood and Pulakat, 2015; Sharma et al., 2020; Gavini et al., 2021), although data for the AT_2R affinity of NP-6A4 are not available.

3. From Peptides to Small Druglike Molecules. Truncation of the three amino acid residues in the N-terminal of Ang II and subsequent N-acetylation delivered the AT_2R selective pentapeptide A, with a K_i value of 37.9 nM. Further structural elaborations, including replacement of the C-terminal phenylalanine for an isoleucine residue, allowed identification of the selective agonist B with a K_i of 0.5 nM and characterized by the hydroxyphenyl moiety, an imidazole ring, a lipophilic side chain, and a C-terminal carboxy group (Georgsson et al., 2006). Even shorter fragments lacking the Tyr or Tyr mimicking residues have been reported, but it is not clear whether these AT_2R selective ligands that demonstrate a more than 10-fold lower AT_2R affinity than B act as agonists or not (Georgsson et al., 2007). As deduced from modeling relying on the structure of the reported AT_2R/sarile complex (Asada et al., 2018), it is suggested that the short peptides A and B depicted in Fig. 7, which both induce neurite outgrowth in NG108-15 cells, adopt a very similar conformation as the AT_2R agonist C21, originally derived from the nonselective AT_1R/AT_2R ligand L-162,313 (Vasile et al., 2020).

TABLE 1
Angiotensin receptor ligands

Compounds	Preparations: AT_1R; AT_2R	Radioligand Assay Conditions	AT_1R K_i (A) or IC_{50} Values (B) (nM)[e]	AT_2R K_i (A) or IC_{50} Values (B) (nM)	AT_2R-Fold Selectivity	References
Historical ATR Subtype Elucidation from Seminal Publications						
CGP42112A	Rat VSMC; human uterus[b]	^{125}I-Ang II; membranes	1750 (A)	0.45 (A)	3889	(Whitebread et al., 1989)
Losartan (EX 89)	Rat VSMC; human uterus[b]	^{125}I-Ang II; membranes	26.2 (A)	100,0000 (A)	3817 (AT_1R)	(Whitebread et al., 1989)
Ang III	Rat VSMC; human uterus[b]	^{125}I-Ang II; membranes	1.95 (A)	0.38 (A)	5.1	(Whitebread et al., 1989)
EXP655 (PD123319 analog)	Rat adrenals; 2-site fit[c]	^{125}I-Ang II; microsomes	100,000 (B)	100 (B)	1000	(Chiu et al., 1989)
Losartan (DuP753)	Rat adrenals; 2-site fit	^{125}I-Ang II; microsomes	17 (B)	100,000 (B)	5882 (AT_1R)	(Chiu et al., 1989)
Ang II	Rat liver; rabbit uterus[b]	^{3}H-Ang II; membranes	1.02 (B)	2.50 (B)	0.4	(Dudley et al., 1990)
Ang III	Rat liver; rabbit uterus[b]	^{3}H-Ang II; membranes	5.57 (B)	1.74 (B)	3.3	(Dudley et al., 1990)
PD123319	Rat liver; rabbit uterus[b]	^{3}H-Ang II; membranes	>10,000 (B)	21.2 (B)	472	(Dudley et al., 1990)
Losartan (Dup753)	Rat liver; rabbit uterus[b]	^{3}H-Ang II; membranes	7.52 (B)	>10,000 (B)	1330 (AT_1R)	(Dudley et al., 1990)
Ang II	Rat adrenals; 2-site fit[d]	^{125}I-Ang II; membranes	0.89 (B)	0.73 (B)	1.2	(Chang and Lotti, 1990)
Ang III	Rat adrenals; 2-site fit[d]	^{125}I-Ang II; membranes	1.3 (B)	0.11 (B)	11.8	(Chang and Lotti, 1990)
WL-19 (PD121981)	Rat adrenals; 2-site fit[d]	^{125}I-Ang II; membranes	(AT_1R blocked)	19 (B)		(Chang and Lotti, 1990)
Losartan (DuP753)	Rat adrenals; 2-site fit[d]	^{125}I-Ang II; membranes	24 (B)	(AT_2R blocked)		(Chang and Lotti, 1990)
PD123319	Rat adrenals	^{125}I-Ang II; membranes	>10,000 (B) (negligible)	34 (B)	294	(Blankley et al., 1991)
Ang II	Rat liver, PC12W cells	^{125}I-Sar^{1}-Ile^{8}-Ang II; membranes	2.6 (B)	2.1 (B)	1.2	(Speth and Kim, 1990)
Losartan	Rat liver, PC12W cells	^{125}I-Sar^{1}-Ile^{8}-Ang II; membranes	54.8 (B)	271,000 (B)	4945 (AT_1R)	(Speth and Kim, 1990)
4-amino-Phe^{6}-Ang II	Rat liver, PC12W cells	^{125}I-Sar^{1}-Ile^{8}-Ang II; membranes	8,637 (B)	12.2 (B)	708	(Speth and Kim, 1990)
Nonselective Synthetic ATR Ligands						
Sar^{1}-Ile^{8}-Ang II (Sarile)	Rat VSMC; human uterus[b]	^{125}I-Ang II; membranes	0.64 (A)	0.45 (A)	1.4	(Whitebread et al., 1989)
Sar^{1}-Ile^{8}-Ang II (Sarile)	Rat adrenals; 2-site fit[d]	^{125}I-Ang II; membranes	0.18 (B)	0.18 (B)	1	(Chang and Lotti, 1990)
Sar^{1}-Ile^{8}-Ang II (Sarile)	Rat liver; rabbit uterus[b]	^{3}H-Ang II; membranes	7.8 (B)	3.48 (B)	2.2	(Dudley et al., 1990)
Sar^{1}-Ile^{8}-Ang II (Sarile)	Rat VSMC; human uterus[b]	^{125}I-Ang II; membranes	0.65 (A)	0.14 (A)	4.6	(Criscione et al., 1990)
Sar^{1}-Val^{5}-Ile^{8}-Ang II (Saralasin)	Rat liver; rabbit uterus[b]	^{3}H-Ang II; membranes	1.69 (B)	1.32 (B)	1.3	(Dudley et al., 1990)
Sar^{1}-Val^{5}-Ile^{8}-Ang II (Saralasin)	Rat VSMC; human uterus[b]	^{125}I-Ang II; membranes	0.22 (A)	0.38 (A)	0.6	(Criscione et al., 1990)
L-162,313	Rat liver, pig uterus	^{125}I-Ang II; membranes	3.9 (A)	2.8 (A)	1.3	(Wan et al., 2004a)
Endogenous Ang Peptides and Selective AT_2R Ligands						
4-amino-Phe^{6}-Ang II	Rat liver, pig uterus	^{125}I-Ang II; membranes	>10,000 (A)	1.4 (A)	7143	(Johannesson et al., 2004)
4-amino-Phe^{6}-Ang II	Rat liver, pig uterus	^{125}I-Ang II; membranes	>10,000 (A)	0.9 (A)	11,111	(Rosenström et al., 2005)
CGP42112A	Rat VSMC; human uterus[b]	^{125}I-Ang II; membranes	1,760 (A)	0.24 (A)	7333	(Criscione et al., 1990)
CGP42112A	Rat AT_1R; AT_2R-HEK-293 cells	^{125}I-Sar^{1}-Ile^{8}-Ang II; whole cells	>10,000 (B)	0.23 (B)	42,863	(Bosnyak et al., 2011)
CGP42112A	Rat AT_1R; AT_2R-HEK-293 cells	^{125}I-Ang II, whole cells	2,700 (B)	0.56 (B)	4821	(Magnani et al., 2014)
CGP42112A	Rat AT_1R; AT_2R-HEK-293 cells	^{125}I-Sar^{1}-Ile^{8}-Ang II; whole cells	2,370 (B)	0.13 (B)	18,136	(Del Borgo et al., 2015)
Ang II	Rat AT_1R; AT_2R-HEK-293 cells	^{125}I-Sar^{1}-Ile^{8}-Ang II; whole cells	7.92 (B)	0.52 (B)	15	(Bosnyak et al., 2011)
Ang II	Rat AT_1R; AT_2R-HEK-293 cells	^{125}I-Ang II; whole cells	3.29 (B)	0.65 (B)	5.0	(Jones et al., 2011)
Ang II	Rat AT_1R; AT_2R-HEK-293 cells	^{125}I-Sar^{1}-Ile^{8}-Ang II; whole cells	1.85 (B)	0.49 (B)	4	(Del Borgo et al., 2015)

(*continued*)

TABLE 1—*Continued*

Compounds	Preparations: AT_1R; AT_2R	Radioligand Assay Conditions	AT_1R K_i (A) or IC_{50} Values (B) (nM)[e]	AT_2R K_i (A) or IC_{50} Values (B) (nM)	AT_2R-Fold Selectivity	References
Ang III	Rat AT_1R; AT_2R-HEK-293 cells	^{125}I-Sar^1-Ile^8-Ang II; whole cells	21.1 (B)	0.65 (B)	33	(Bosnyak et al., 2011)
Ang III	Rat AT_1R; AT_2R-HEK-293 cells	^{125}I-Sar^1-Ile^8-Ang II; whole cells	4.29 (B)	0.29 (B)	15	(Del Borgo et al., 2015)
Ang-(1-7)	Rat AT_1R; AT_2R-HEK-293 cells	^{125}I-Sar^1-Ile^8-Ang II; whole cells	>10,000 (B)	246 (B)	41	(Bosnyak et al., 2011)
Ang-(1-7)	Rat AT_1R; AT_2R-HeLa cells	^{125}I-Ang II, whole cells	724 (A)	95.5 (A)	7.6	(Flores-Muñoz et al., 2011)
Ang IV	Rat AT_1R; AT_2R-HEK-293 cells	^{125}I-Sar^1-Ile^8-Ang II; whole cells	>10,000 (B)	48.6 (B)	206	(Bosnyak et al., 2011)
Ang-(1-9)	Rat AT_1R; AT_2R-HeLa cells	^{125}I-Ang II, whole cells	245 (A)	525 (A)	0.47	(Flores-Muñoz et al., 2011)
β-Ile^5-Ang II	Rat AT_1R; AT_2R-HEK-293 cells	^{125}I-Ang II; whole cells	>10,000 (B)	10.6 (B)	955	(Jones et al., 2011)
β-Pro^7-Ang III	Rat AT_1R; AT_2R-HEK-293 cells	^{125}I-Sar^1-Ile^8-Ang II; whole cells	>10,000 (B)	0.47 (B)	21,377	(Del Borgo et al., 2015)
Tyr^6-Ang II	Rat AT_1R; AT_2R-HEK-293 cells	^{125}I-Ang II; whole cells	72,000 (B)	4.0 (B)	18,000	(Magnani et al., 2014)
Novokinin	Human AT_1R-CHO cells; human AT_2R HeLa cells	^{125}I-Sar^1-Ile^6-Ang II; ^{125}I-CGP42112A, membranes	685,000 (A)	7,350 (A)	93	(Yamada et al., 2008a)
NP-6A4			?	?		(Toedebusch et al., 2018)
C21	Rat liver, pig uterus	^{125}I-Ang II; membranes	>10,000 (A)	0.4 (A)	25,000	(Wan et al., 2004b)
C21	Rat AT_1R; AT_2R-HEK-293 cells	^{125}I-Sar^1-Ile^8-Ang II; whole cells	>10,000 (B)	2.29 (B)	4367	(Bosnyak et al., 2011)
C21	Rat AT_1R; AT_2R-HEK-293 cells	^{125}I-Sar^1-Ile^8-Ang II; whole cells	>10,000 (B)	1.47 (B)	6803	(Isaksson et al., 2019)
PD123319[a]	Rat AT_1R; AT_2R-HEK-293 cells	^{125}I-Sar^1-Ile^8-Ang II; whole cells	>10,000 (B)	5.6 (B)	1786	(Bosnyak et al., 2011)
PD123319[a]	Rat AT_1R; AT_2R-HEK-293 cells	^{125}I-Ang II; whole cells	>10,000 (B)	8.32 (B)	1202	(Jones et al., 2011)
PD123319[a]	Rat AT_1R; AT_2R-HEK-293 cells	^{125}I-Sar^1-Ile^8-Ang II; whole cells	>10,000 (B)	3.12 (B)	3205	(Del Borgo et al., 2015)
PD123319[a] (EMA200)	Rat AT_1R; AT_2R-HEK-293 cells	^{125}I-Sar^1-Ile^8-Ang II; membranes	210,500 (B)	71.7 (B)	2935	(Smith et al., 2013b)
EMA401[a]	Rat AT_1R; AT_2R-HEK-293 cells; human AT_1R-CHO cells; human AT_2R HeLa cells	^{125}I-Sar^1-Ile^8-Ang II; membranes^{125}I-Sar^1-Ile^8-Ang II; ^{125}I-CGP42112A, whole cells	408,000 (B); not calculable	39.5 (B); 39 (B)	10,329; >10,000	(Smith et al., 2013b)
C38[a]	Rat liver, pig uterus	^{125}I-Ang II; membranes	>10,000 (A)	19 (A)	526	(Murugaiah et al., 2012)
C38[a]	Rat AT_1R; AT_2R-HEK-293 cells	^{125}I-Sar^1-Ile^8-Ang II; whole cells	>10,000 (B)	694 (B)	14.4	(Isaksson et al., 2019)

[a] AT_2R antagonist.
[b] Other tissues tested.
[c] AT_1R site blocked for AT_2R determination.
[d] Respective sites blocked by ATR-selective compound.
[e] Some IC_{50} values at AT_1R were estimated (>10,000) due to poor AT_1R binding.

B. Nonpeptide Agonists

1. Compound 21. The nonselective thiophene derivative L-162,313, exhibiting IC_{50} values of 1.1 nM (AT_1R) and 2.0 nM (AT_2R), respectively served as a starting point in the design of C21 (Fig. 7). L-162,313 was the first nonpeptide low-molecular-weight compound reported to act as an agonist at the AT_1R (Kivlighn et al., 1995; Perlman et al., 1995). Likewise, the corresponding biphenyl derivative acts as a nonselective AT_1R agonist with IC_{50} values of 2.1 nM (AT_1R) and 0.7 nM (AT_2R), respectively. Notably, a subtle molecular alteration of the structure of the latter ligand can determine the agonist/antagonist properties. Hence, elimination of one single methyl group from the isobutyl side chain resulted in a ligand demonstrating AT_1R antagonism (Perlman et al., 1997). Subsequently L-162,313 was also reported to act as an AT_2R agonist in vivo (Wan et al., 2004a). By introduction of a bicyclic benzimidazole to replace the imidazopyridine scaffold of L-162,313, the affinity for AT_2R was essentially retained but a drastic drop of affinity for AT_1R could be achieved. Further stepwise structural manipulations eventually provided C21. Indeed, the AT_2R-selective imidazole derivative C21 demonstrated high affinity for the AT_2R (AT_2R; $K\acute{C}_i$ = 0.4 nM) but not for the AT_1R (K_i > 1000 nM) (Wan et al., 2004b). For a recent review on the medicinal chemistry related to the discovery of C21,

see Hallberg et al. (2018). Due to its favorable pharmacokinetic properties (oral bioavailability; 4–6 hours terminal half-life), C21 has become the most frequently used agonist in AT_2R research and is currently being tested in phase II and phase III clinical trials, as described in Section VII.

The structural information on the AT_2R/sarile complex (Asada et al., 2018) combined with previous modeling (Sallander et al., 2016) and subsequent molecular dynamic simulations allowed a proposal of a tentative binding mode of C21 binding to AT_2R (Vasile et al., 2020). C21 binds deeper in the transmembrane bundle of AT_2R than sarile, but the C-terminal carboxy group of the peptides and the acidic sulfonyl carbamate group of C21 demonstrate analogous electrostatic interactions with the positively charged $K215^{5.42}$ and $R182^{4.64}$. Moreover, a hydrogen bond between NH of the sulfonyl carbamate and $T125^{3.33}$ is created. The modeling of C21 in AT_2R reveals that the aliphatic chain of the butyloxy substituent is extending parallel to the $K215^{5.42}$ sidechain. Furthermore, the isobutyl group of C21 is occupying a hydrophobic cavity formed by residues from TM2, TM3, TM6, and TM7. The imidazole ring forms a hydrogen bond with $Y104^{2.65}$ and thereby provides a second electrostatic anchoring point of importance. Analogous hydrogen bonds to $Y104^{2.65}$ are formed with all AT_2R-selective peptide agonists examined according to modeling and the molecular dynamics simulations (Vasile et al., 2020).

2. Other Molecules of the C21 Family. A tetrazole ring rather than the sulfonylcarbamate as a carboxylic acid bioisostere results in an AT_2R-selective ligand C, exhibiting a considerably lower affinity (AT_2R K_i value of 189 nM) (Wu et al., 2006) (Fig. 8). On the contrary, a remarkable acceptance of groups replacing the imidazole ring was observed. For example, the N-pyrrolidine derivative D devoid of a heteroaromatic group shows a K_i value of 3.5 nM and acts as an AT_2R agonist in the NG108-15 cell neurite outgrowth assay. This ligand and other related ligands are, in comparison with C21, considerably less prone to inhibit a panel of enzymes from the CYP family (e.g., 3A4 and 2C9) (Mahalingam et al., 2010). Furthermore, regarding the biaryl scaffold, the thiophene ring of the ligands in the C21 series can in general be replaced by a benzene ring with only minor impact on either AT_2 R affinity or AT_1R/AT_2R selectivity (Liu et al., 2013). However, there are exceptions, and in the case of the benzamide E that exhibits a K_i value of 3.0 nM, a replacement with a biphenyl scaffold afforded a ligand with a 50-fold lower AT_2R affinity (K_i = 145 nM) (Wallinder et al., 2008) (Fig. 8). The furanyl derivative F, acting as an agonist in the NG108-15 cell neurite outgrowth assay, demonstrated moderate AT_2R affinity (K_i = 9.1 nM), contrary to the corresponding 2,4 substituted thiophene and the 2,5 substituted pyridine derivatives, which somewhat surprisingly showed negligible AT_2R affinity (Murugaiah et al., 2007). Selective high affinity AT_2R ligands could be obtained when a phenylthiazole displaced the phenylthiophene scaffold (Gopalan et al., 2022) and with substituted benzimidazoles displacing the imidazole ring (Roy et al., 2022). Furthermore, the biphenyl derivative G with a 1-3 substitution (*meta*) pattern acts as an agonist in the NG108-15 cell assay and exhibits a K_i value of 8.2 nM (Wallinder et al., 2014).

C. Nonpeptide Antagonists

1. C38 (M132) and Analogs. Migration of the methylene imidazole group of C21 from the *para* position to the *meta* position afforded C38 (also called M132) (AT_2R; K_i = 19 nM) that did not act as an agonist but an antagonist in the NG108-15 neurite outgrowth cell assay (Murugaiah et al., 2012; Guimond et al., 2013). Notably, C38 and its regioisomer, the agonist C21, exhibit similar effects on NO production in lipopolysaccharide (LPS)-treated mouse macrophage cell lines, suggesting that C38 is a partial AT_2R agonist in this macrophage system (Isaksson et al., 2020). A large series of C38 analogs with a *meta* substitution pattern was subsequently prepared and examined (Wannberg et al., 2018; Isaksson et al., 2019). The AT_2R agonist G, but not C38, can adopt a conformation with an extended distance between imidazole ring and the lipophilic side chain, which according to modeling should allow activation of the AT_2R (Wallinder et al., 2014, 2019) (Fig. 8). Recently, a related example of an AT_2R ligand with a *meta* substitution pattern and comprising a tether between the imidazole ring and the scaffold was reported. The latter ligand acted as an agonist with a K_i of 9.3 nM and caused an AT_2R-mediated concentration-dependent vasorelaxation of precontracted mouse aorta (Wannberg et al., 2021).

2. PD123319/PD123177. The AT_2R-selective peptide agonist CGP42112A and the two AT_2R antagonists PD123319 and PD123177 were developed at the same time as losartan, which was the first AT_1R antagonist available clinically. These two prototype AT_2 R antagonists belong to a family of compounds with several members that are structurally very different from the AT_1R antagonists (e.g., losartan) and the AT_2R agonists (e.g., C21) (Fig. 8). PD123319 was first described (Dudley et al., 1991) to potently inhibit tritiated Ang II binding in rabbit uterine homogenates (IC_{50} value ~21nM) and inhibit iodinated Ang II or Sar-Ile-Ang II in R3T3 cells (IC_{50} value ~30nM) at a binding site later to be called the AT_2R. PD123319 exhibited 5- to 10-fold greater AT_2R affinity than PD123177 (IC_{50} values 130–288nM) under the same conditions (Dudley et al., 1990; Dudley and Summerfelt, 1993). PD123319 has been widely used to confirm

AT_2R involvement in a majority of studies elucidating the functional effects of C21 and other AT_2R agonists. However, there are questions about the AT_2R selectivity of PD123319 that, as mentioned earlier, has recently been reported to be a competitive antagonist at the MAS-related G-protein–coupled receptor D (MrgD) (Lautner et al., 2013). In many instances, the effects of Ang-(1-7) and Ang-(1-9) were inhibited by PD123319, which is consistent with the AT_2R affinity of these endogenous peptides (see Section IV.A.1.c/d). Indeed, PD123319 is used almost exclusively to implicate an involvement of AT_2R function. In addition, PD123319 has been reported to evoke AT_2R agonistic-like effects in a number of experimental paradigms, including a hyperoxic lung injury model in which it also acted as an antagonist (Wagenaar et al., 2014). A potential agonistic effect was also seen in a model of rat colitis (Zizzo et al., 2017, 2020). The apparent partial agonistic properties of PD123319 and C38 (see above) can thus create problems in interpreting functional data.

3. EMA401 and Analogs. Modification of the prototype selective AT_2R antagonist PD123319 afforded the high-affinity AT_2R ligand EMA401 (IC_{50} = 39 nM), expected to be an antagonist and shown in preclinical studies to exert analgesic properties in neuropathic pain (Rice et al., 2014; Smith and Muralidharan, 2015; Smith et al., 2016). However, it was never clarified if the beneficial effects of EMA401 were related to AT_2R agonist or antagonist functions (see Section VI.F.11). Such preclinical studies led to phase II studies to examine the analgesic efficacy and safety of EMA401 in patients with postherpetic neuralgia and painful diabetic neuropathy, as discussed in Section VII.A (Rice et al., 2021). However, clinical development of EMA401 has been terminated due to preclinical hepatic toxicity.

Key points related to Section IV on AT_2R-selective ligands are:

- Endogenous angiotensin peptides bind to AT_2R. Ang II, as the well accepted main effector of the RAS, is equipotent and effective at both AT_1R and AT_2R, although AT_1R actions predominate due to ubiquitous AT_1R distribution. Ang III may be the main endogenous ligand for AT_2R, at least in kidney.
- Synthetic peptide derivatives exhibit marked AT_2R selectivity. CGP42112 was the first reported highly AT_2R-selective ligand and was a key tool for the classification of Ang receptors into AT_2R and AT_1R. Subtle modifications to Ang II, exemplified by p-amino-Phe^6-Ang II, or to Ang III, exemplified by β-Pro^7-Ang III, have resulted in peptides with marked AT_2R selectivity. A number of ligands are reported to be AT_2R agonists but many lack binding data to match function or vice versa.
- Small molecule (nonpeptide) compounds are providing opportunities for clinical translation. Hallberg's group was instrumental in synthesizing many series of nonpeptide AT_2R ligands, culminating in the first orally active small-molecule AT_2R agonist, C21, which has contributed enormously to elucidating the protective role of AT_2R in (patho)physiology and is currently in idiopathic pulmonary fibrosis and COVID-19 trials. The development of newer AT_2R agonists as well as antagonists is likely to be an active area of research that will continue to define AT_2R pharmacology.

V. AT_2-Receptor Physiology

An early view of the potential whole-body functions of AT_2R was gained from studies in which the *Agtr2* was deleted from mice (Hein et al., 1995a; Ichiki et al., 1995). Although neither study reported any changes in morphology or mortality of the AT_2R-KO mice versus wild-type animals, both studies reported a significant reduction in locomotion and exploratory behavior consistent with the normal location of AT_2R in the locus coeruleus in the brain (Tsutsumi and Saavedra, 1992). One of the studies reported a decrease in drinking behavior after water deprivation in the AT_2R-KO mice, possibly a consequence of the decreased exploratory behavior (Hein et al., 1995a). Other phenotypes apparent in the AT_2R-KO mice were reduced body temperature and increased basal BP (Ichiki et al., 1995) and in both studies an enhancement of Ang II-induced increases in BP. The potential reasons for any discrepancies in phenotypes of the AT_2R-KO mice are further discussed in Section VIII, *Open Questions in AT_2R Research*. These landmark studies provided an initial indication that AT_2 Rs can exert effects at the whole-organism level. Subsequent studies have used an array of approaches to uncover AT_2R-induced effects in normal animals, as discussed in the following paragraphs.

A. Natriuresis/Diuresis

AT_2Rs play an important role in the regulation of kidney function, especially Na^+ and water excretion. Early studies showed that N(G)-nitro-L-arginine methyl ester (L-NAME) pretreated rats have blunted pressure-natriuresis and diuresis curves and that AT_2 R blockade with PD123319 shifts these curves backward toward control levels, indicating that the excretory impairment produced by NO synthesis blockade is at least partially dependent on the activation of AT_2R (Madrid et al., 1997). AT_2R-KO demonstrate pressor and antinatriuretic hypersensitivity to Ang II infusion, indicating that pressure-natriuresis is shifted to the right (less sensitive) in the absence of AT_2Rs (Siragy et al., 1999). Furthermore, AT_2R-KO

animals have markedly reduced baseline and Ang II-stimulated renal BK and cyclic GMP (cGMP) levels, suggesting that AT_2Rs play a counterregulatory protective role, mediated by BK and NO, against the antinatriuretic actions of Ang II via AT_1Rs (Siragy et al., 1999). Subsequent studies in B_2R receptor-KO mice confirmed that NO can be produced by two alternative pathways: directly via the AT_2R or indirectly via AT_2R stimulation of BK production and activation of the B_2R receptor (Abadir et al., 2003). More detailed analyses of the pressure-natriuresis relationship confirmed the rightward shift in AT_2R-KO animals without a change in glomerular filtration rate, indicating an action predominantly via changes in tubule Na^+ reabsorption with a possible contribution from reduced renal blood flow (Gross et al., 2000; Obst et al., 2003). Similar to AT_1Rs, however, renal AT_2Rs also inhibit intrarenal RAS activity through direct inhibition of renin release from renal juxtaglomerular cells and consequently reduced Ang II formation (Siragy et al., 2005, 2007).

The aforementioned pressure-natriuresis studies in AT_2R-KO animals suggested the possibility that AT_1R blockade induces natriuresis by activation of AT_2Rs. In a normal (Sprague-Dawley) rat model, the natriuretic effect of intrarenal AT_1R blockade was abolished by concurrent AT_2R inhibition (Padia et al., 2006). Although the endogenous agonist for renal Ang receptors had been assumed to be Ang II, the octapeptide does not induce natriuresis at any physiological infusion rate and the preferred endogenous AT_2R agonist was initially identified as Ang II metabolite des-aspartyl[1]-Ang II (Ang III) (Padia et al., 2006). Ang III was subsequently confirmed as the major peptide agonist for AT_2R-induced natriuresis in a series of studies using inhibitors of aminopeptidase A, which forms Ang III from Ang II, and/or aminopeptidase N, which metabolizes Ang III to smaller nonfunctional peptide fragments (Padia et al., 2007, 2008; Kemp et al., 2012). Renal dopamine D_1Rs as well as AT_2R are important natriuretic receptors counterbalancing AT_1R-mediated tubular Na^+ reabsorption, and their interactions are mutually cooperative and interdependent (Gildea et al., 2012). Dopamine-induced natriuresis via D_1R requires AT_2R activation (Salomone et al., 2007), and AT_2R recruitment along microtubules to the apical plasma membranes of renal proximal tubule cells (RPTCs) occurs by an adenylyl cyclase/cAMP/protein kinase C-dependent pathway (Padia et al., 2012). In addition to D_1R, dopamine D_3 receptors and AT_2Rs are synergistic in producing natriuresis and diuresis (Yang et al., 2015).

Sex differences have been identified in AT_2R-induced natriuresis, possibly due to the localization of the *Agtr2* gene on the X chromosome. The pressure-natriuresis curve is shifted to the left (more sensitive) in females compared with males, and AT_2R activation in females attenuates enhanced Ang II-dependent resetting of renal tubuloglomerular feedback via AT_1Rs (Hilliard et al., 2011; Brown et al., 2012). However, AT_2R activation increases Na^+ and water excretion equally in males and females (Hilliard et al., 2012; Kemp et al., 2014). Sex differences in the chronic pressure-natriuresis relationship may be due at least in part to increased renal AT_2R expression in females compared with males (Mirabito et al., 2014), although this requires further study.

The natriuretic response to AT_2R activation is largely, if not exclusively, a renal proximal tubule (RPT) event with a possible small contribution from the thick ascending limb of the loop of Henle (Herrera and Garvin, 2010; Kemp et al., 2014). AT_2Rs reduce AT_1R expression and function by a NO/cGMP/Sp1-dependent mechanism (Yang et al., 2012). AT_2R activation is accompanied by translocation of the receptor to the apical brush borders of RPTCs (a mechanism thought to enhance and sustain the natriuretic response) and internalization of major RPTC Na^+ transporters Na^+-H^+ exchanger-3 (NHE-3) and Na^+/K^+-ATPase (NKA) in a BK/NO/cGMP-dependent manner (Kemp et al., 2014). In Ang II infused rats, chronic AT_2R activation initiates and sustains receptor translocation to RPTC apical membranes, prevents Na^+ retention resulting in a negative Na^+ balance, and lowers BP (Kemp et al., 2016). Activating renal AT_2Rs with C21 engenders the physical association of AT_2Rs with PP2A heterotrimer AB55αC and increases renal PP2A activity (Kemp et al., 2022). The AT_2R/PP2A complex translocates to the apical plasma membranes of RPTCs. C21-induced natriuresis, renal cyclic GMP formation, and AT_2R/PP2A translocation to apical plasma membranes are abolished by coadministration of PP2A inhibitor calyculin A (Kemp et al., 2022). The intracellular signaling mechanisms by which AT_2R activation induces natriuresis in RPTC are summarized in Fig. 9.

AT_2R-induced natriuresis is defective in both hypertensive and prehypertensive SHR, an Ang II-dependent model of human hypertension (Kemp et al., 2019, 2020). This may account at least in part for increased Ang II-dependent Na^+ reabsorption in SHR. The AT_2R defect in SHR is a primary receptor defect and not due to increased metabolism of AT_2R agonist Ang III (Kemp et al., 2020). Signaling pathways involving D_1R [cAMP/protein kinase A (PKA)] and AT_2R [cGMP/protein kinase G (PKG)] translocation to apical plasma membranes converge at PP2A (Gildea et al., 2019), and in SHR AT_2R signaling to PP2A is defective. This defect likely leads to Na^+ retention and hypertension by allowing unopposed AT_1R-mediated renal Na^+ transport in this animal model of human hypertension (Kemp et al., 2022).

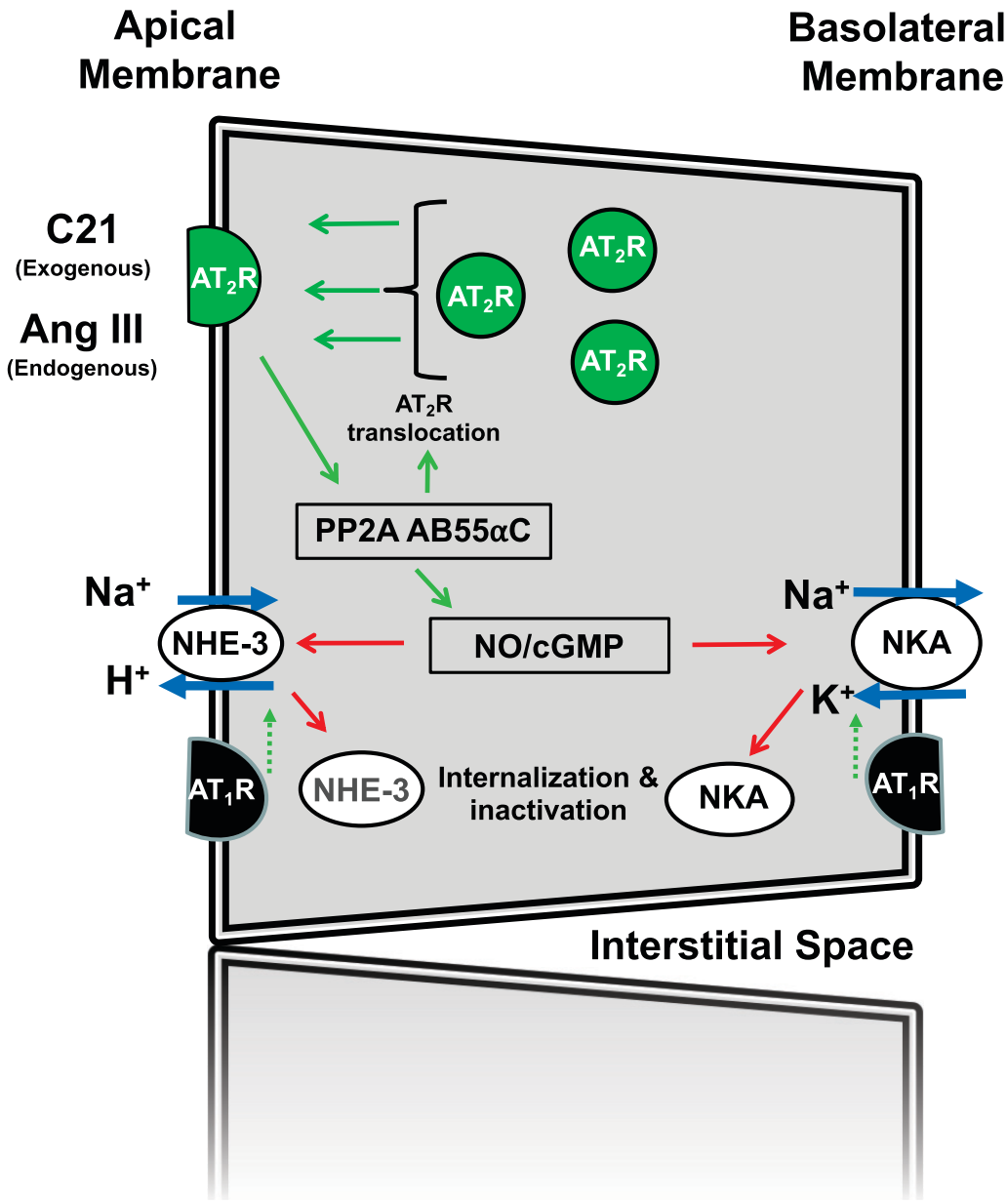

Fig. 9. Renal proximal tubule cell AT_2R/PP2A AB55αC signaling pathway by which receptor activation increases cyclic GMP (cGMP) production, inhibits its Na^+ reabsorption, and induces natriuresis. Green arrows indicate stimulation, red arrows depict internalization and inactivation, red lines depict inhibition, blue arrows stand for effects on ion exchanges by Na^+ transporters, and dashed arrows indicate impaired/reduced responses. AT_2R activation by exogenous nonpeptide agonist C21 stimulates AT_2R recruitment from intracellular sites to the apical plasma membranes of renal proximal tubule cells via PP2A-dependent signaling, reinforcing, and sustaining the natriuretic response. AT_2R activation via a PP2A AB55αC signaling pathway increases cGMP production, which internalizes and inactivates major Na^+ transporter molecules Na^+-H^+-exchanger-3 (NHE-3) and Na^+-K^+-ATPase (NKA), counterbalancing AT_1R actions to increase Na^+ reabsorption by stimulating these transporters.

RPTC AT_2Rs are especially effective in stimulating natriuresis/diuresis in the obese Zucker rat, a model of obesity, insulin resistance, and mild hypertension in humans. AT_2Rs are upregulated in these rats compared with lean controls and mediate the natriuretic/diuretic effects of AT_1R blockade (Hakam and Hussain, 2005). The enhanced natriuresis in this model is accompanied by inhibition of NKA via the NO/cGMP pathway in RPTCs (Hakam and Hussain, 2006). AT_2Rs play a protective role against increased BP in obese Zucker rats, which is due to their renin-suppressive action (Siragy et al., 2005; Siddiqui et al., 2009; Ali and Hussain, 2012; Ali et al., 2013, 2015).

Overall, renal AT_2Rs acting in tandem with dopamine receptors counterbalance Na^+ retention elicited by Ang II via AT_1Rs. Because AT_2Rs inhibit Na^+ transport in the RPT where the majority of Na^+ is reabsorbed and no effective RPT natriuretic/diuretic agent is currently available or approved for clinical use, AT_2R agonists appear to be excellent pharmacologic candidates for treatment of disorders of Na^+/fluid retention, as expanded upon in Section VI.A.9.

B. Cardiovascular Regulation by the Central Nervous System

1. Lowering of Blood Pressure. There is much evidence that activation of AT_2Rs within the brain decreases BP in normal animals (Steckelings et al., 2017b; Assersen et al., 2020). These effects are consistent with the demonstration that AT_2Rs are located on neurons within or adjacent to CNS areas that play a major role in regulating sympathetic outflow and baroreflex sensitivity, as noted in Section III.E.6 (Nuyt et al., 2001; de Kloet et al., 2016b; Sumners et al., 2020). The initial evidence that stimulation of brain AT_2Rs lowers BP was *indirect*, specifically that the AT_1R-mediated pressor responses after intracerebroventricular (i.c.v.) injection of Ang II were potentiated by coapplication of the AT_2R antagonist PD123319 (Li et al., 2003). More *direct* evidence for BP lowering actions of brain AT_2Rs has come from both pharmacological and gene transfer approaches. Two studies using the pharmacological approach provided such direct evidence for brain AT_2R involvement. One of these studies demonstrated that chronic intracerebroventricular infusion of C21 produced sympathoinhibition and decreased BP in rats (Gao et al., 2011), and the other revealed that intracerebroventricular infusion of GGP42112 enhanced the renal sympathoinhibitory response to volume expansion (Abdulla and Johns, 2017), providing important evidence for cardiovascular actions of brain AT_2Rs. However, intracerebroventricular administration does not reveal the specific loci of AT_2R agonist effects. Other studies employed more targeted administration of either CGP42112A or C21 into cardiovascular control centers and demonstrated that AT_2Rs in both the RVLM and the IML are important mediators of the sympathoinhibitory and depressor actions (Gao et al., 2008b; Chao et al., 2013; Légat et al., 2017); on the other hand, acute local infusion of C21 in the NTS failed to alter BP (Légat et al., 2019). A role for RVLM AT_2R in lowering BP was also suggested by gene transfer studies. Gao et al., 2008a demonstrated that adenoviral-mediated overexpression of AT_2R at the RVLM lowered BP and decreased circulating norepinephrine levels in normotensive rats. Two recent studies have further refined the knowledge of how CNS AT_2Rs lower BP by defining the particular neuronal phenotypes and transmitters involved. First, Légat et al. (2017) found that the depressor response to C21 is mediated by stimulation of GABA release within the RVLM and subsequent $GABA_a$ receptor-mediated decreases in sympathetic tone. In a second study, Mohammed et al. (2021) demonstrated that AT_2Rs located on GABA neurons within the NTS are critical for the depressor action of C21 chronically infused via the intracerebroventricular route. Stimulation of AT_2R on GABA neurons attenuated GABA signaling and in doing so lifted the tonic inhibition of baroreflex pathways provided by these GABAergic neurons, consequently reducing BP. In summary, activation of brain AT_2Rs in normotensive animals can lower BP, as summarized in Fig. 10, though a full picture of the neuronal circuitry and mechanisms involved remains to be established.

2. Inhibition of Vasopressin Secretion. Evidence also exists that activation of brain AT_2Rs inhibits vasopressin (AVP) secretion. Nerve terminals and processes that arise from GABA neurons surrounding the PVN contain AT_2Rs and synapse with AVP neuron cell bodies in the PVN (de Kloet et al., 2016b). Stimulation of these AT_2Rs by C21 led to a GABA-dependent decrease in activity of AVP neurons within the PVN and eventually decreased plasma AVP levels (de Kloet et al., 2016a). Whether this reduction in AVP levels lowers BP is yet to be determined.

C. Regulation of Vascular Diameter

Given the ubiquitous expression of AT_1Rs, Ang II-mediated vasoconstriction prevails over any potential counterregulatory effects of vascular AT_2R function. However, the first clues that Ang II itself may exert a (masked) vasodilator component to its action were deduced from observations that the AT_2R antagonist PD123319 may enhance Ang II-mediated contraction (Henrion et al., 2001; Widdop et al., 2003), most notably seen using vessels such as uterine arteries that exhibit prominent AT_2R expression (Zwart et al., 1998; McMullen et al., 1999; Hannan et al., 2003). In addition, analogous data whereby PD123319 potentiated Ang II-induced contraction has been reported for healthy human coronary microarteries (Batenburg et al., 2004) and diseased human radial arteries (Zulli et al., 2014), although a lack of PD123319-induced potentiation of Ang II-evoked contraction was also reported using human radial and internal mammary arteries (van de Wal et al., 2007; Zulli et al., 2014). In a few instances, exogenously administered Ang II alone evoked AT_2R-mediated vasodilation in vitro (Henrion et al., 2001) and in vivo (Sampson et al., 2008, 2012a). On the other hand, there are instances of AT_2R-mediated (PD123319-sensitive) mesenteric contraction that occurred in vessels from SHR or aged rats (Touyz et al., 1999; You et al., 2005; Pinaud et al., 2007). Interestingly, these ex vivo AT_2R-mediated responses were converted from contraction (reduced mesenteric diameter) to relaxation (increased diameter) when resting BP was lowered to normotensive levels in SHR (You et al., 2005), whereas hydralazine or tempol blunted AT_2R-mediated contraction that involved reactive oxygen species (ROS) production in aged vessels (Pinaud et al., 2007). In measuring human forearm blood flow, both AT_2R-mediated constriction and dilation resulting from Ang II infusion were deduced, whereas the selective AT_2R agonist CGP42112A increased flow only (Schinzari et al., 2011).

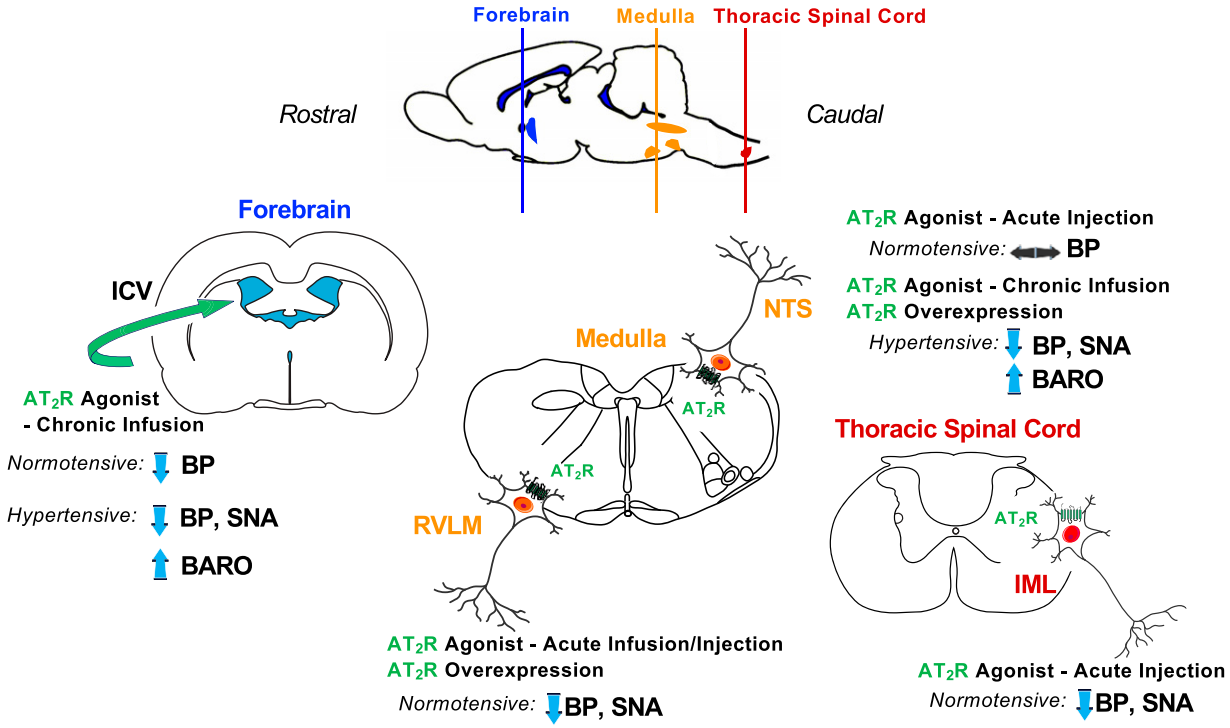

Fig. 10. Summary of the cardiovascular consequences of activation of the AT_2R within the CNS of normotensive or hypertensive rodents. Shown at the top is a sagittal section through a rodent brain; the colored vertical lines represent the brain levels corresponding to the three coronal sections presented below. Forebrain section illustrates the changes in blood pressure (BP), sympathetic nerve activity (SNA), and baroreflex function (BARO) after intracerebroventricular (i.c.v.) infusion of AT_2R agonists; medulla section illustrates the changes in BP, SNA, and BARO resulting from injection/infusion of AT_2R agonists or viral-mediated overexpression of AT_2R at the nucleus of the solitary tract (NTS) or rostral ventrolateral medulla (RVLM); and thoracic spinal cord section shows the changes in BP and SNA after AT_2R agonist injection into the intermediolateral column (IML) of the spinal cord.

AT_1R blockade often unmasked acute AT_2R-mediated vasodilator responses to Ang II using in vitro preparations such as mesenteric arteries after acute (Widdop et al., 2002, 2003; You et al., 2005) or chronic (Widdop et al., 2002; Cosentino et al., 2005; Savoia et al., 2005, 2006) AT_1R blockade, and similar AT_2R-mediated vasodilator effects are noted in vivo (see Section VI.C.4). Likewise, Ang II relaxed human coronary arteries in the presence of acute AT_1R blockade (Batenburg et al., 2004), whereas gluteal arteries obtained by biopsy from diabetic hypertensive patients relaxed to Ang II but only in patients chronically treated with valsartan for 1 year, which correlated with increased AT_2R expression (Savoia et al., 2007).

The work of Henrion's group has identified that endogenous AT_2R activation is critically involved in the control of vascular tone by evoking vasodilation in response to flow (shear stress) that in turn opposes active myogenic tone. This flow-mediated increase in vessel diameter, mediated by endothelial-derived NO, is best described for AT_2R in the mesenteric circulation but is blunted in hypertension (Matrougui et al., 1997, 1999, 2000). Notably, the same group has reported that chronic high-flow dependent vascular remodeling was also AT_2R dependent (see Section VI.C).

The use of selective AT_2R agonists confirmed the importance of AT_2R-mediated vasodilation. CGP42112A increased mesenteric artery diameter in a concentration-dependent manner similarly to Ang II and the vasodilator effects of Ang II were not desensitized, unlike AT_1R-mediated contraction (Widdop et al., 2002). Selective AT_2R agonists such as CGP42112A (Widdop et al., 2002; Baranov and Armstead, 2005; Jones et al., 2011; Del Borgo et al., 2015), C21 (Bosnyak et al., 2010; Yan et al., 2018) or β-Pro^7-Ang III (Del Borgo et al., 2015) relax precontracted vessels, although the vasorelaxant effects of C21 were not always blocked by PD123319 (Verdonk et al., 2012). C21 or β-Pro^7-Ang III dose-dependently increased renal flow to a greater extent in female than male normotensive rats (Hilliard et al., 2012; Krause et al., 2020), and similar effects were seen using C21 in female, but not male, hypertensive rats (Hilliard et al., 2014). Finally, there is general agreement that the majority of vasodilator effects mediated by AT_2R stimulation are mediated by NO and/or BK-cGMP pathways (Henrion et al., 2001; Widdop et al., 2003; Batenburg et al., 2004; Peluso et al., 2018). For example, inhibition of BK by B_2R blockade or kininogen deficiency impaired AT_2R-mediated relaxation of rat mesenteric arteries (Katada and Majima, 2002), consistent with AT_2R stimulation increasing vascular

BK production (Tsutsumi et al., 1999). Conversely, the contribution of vascular BK to flow-dependent vasodilation in mouse carotid arteries relied on the presence of AT_2Rs (Bergaya et al., 2004), demonstrating the reciprocal dependency between AT_2Rs and B_2Rs. In this context, AT_2R-B_2R dimers have been reported that could influence functional outcomes (see Section III.D.5).

D. Intestine

1. Sodium/Glucose Cotransporter-1–Mediated Glucose Absorption. Sodium/glucose cotransporter-1 (SGLT1) is a primary glucose transporter expressed on the brush border membrane of enterocytes. There are three lines of evidence that AT_2Rs can exert stimulatory effects on glucose transport mediated by SGLT1. First, studies using the AT_1R blocker (ARB) losartan demonstrated that it enhances glucose uptake by rat ileal enterocytes (Wong et al., 2007); furthermore, in human jejunal enterocytes, Ang II in the presence of losartan tended to stimulate glucose transport via SGLT1 (Casselbrant et al., 2015). The latter study also demonstrated that Ang II in the presence of the AT_2R antagonist PD123319 inhibited glucose transport via SGLT1, suggesting that AT_2Rs stimulate glucose transport (Casselbrant et al., 2015). This was further confirmed by direct activation of the receptor with the agonist C21 (Casselbrant et al., 2015).

2. Acid, Base Secretion. There is evidence that AT_2Rs can influence stomach acid secretion via CNS mechanisms, as intracerebroventricular administration of novokinin, a low-affinity peptide agonist of the AT_2R, significantly inhibits basal gastric acid secretion (Zhang et al., 2016). This effect of novokinin was sensitive to intracerebroventricular administration of the AT_2R antagonist PD123319 and systemic administration of the prostaglandin synthesis inhibitor indomethacin, suggesting the involvement of an AT_2R-prostaglandin pathway (Zhang et al., 2016). Gastric acid secretion is an important part of the digestive process; however, the acid secreted in the stomach is neutralized by the alkali secreted by the duodenal mucosa, and AT_2Rs have been shown to exert direct effects on alkali secretion. AT_2R activation by Ang II (in the presence of the AT_1R antagonist losartan) or the agonist CGP42112A increased mucosal alkaline secretion by 50%, which is attenuated by PD123319 and B_2R receptor antagonist HOE140, suggesting the role of AT_2R-B_2R pathway (Johansson et al., 2001; Ewert et al., 2003a).

Key Points related to Section V on AT_2R physiology are:

- Under normal conditions, selective AT_2R activation exerts well-described natriuretic and diuretic effects, vasodilation, stimulation of intestinal glucose transport, and lowering of BP (via CNS mechanisms).
- These effects are likely to be a fundamental component of normal homeostatic mechanisms that control Na^+ and fluid balance, in effect keeping in check the mechanisms (e.g., vasoconstriction, antinatriuresis/antidiuresis) that help restore extracellular volume after fluid loss.
- The ability of AT_2R activation to reverse antinatriuresis (and lower BP) in angiotensin-induced increases in BP suggests an important AT_1R counterregulatory role for AT_2Rs in renal physiology.

VI. AT_2-Receptor in Disease

A. Kidney

1. Chronic Kidney Disease. Chronic kidney disease (CKD) is an increasingly burdensome global public health problem. For example, the US National Institute for Diabetes and Digestive and Kidney Diseases estimates that 14% of U.S. adults have CKD (https://www.niddk.nih.gov/health-information/health-statistics/kidney-disease). The leading causes of CKD are diabetes and hypertension, with almost half of individuals with CKD also having diabetes and/or self-reported cardiovascular disease (CVD). Indeed, persons with CKD are at high risk for CVD, and the presence of CKD often complicates CVD treatment and prognosis. Hypertension and proteinuria are the greatest risk factors for CKD progression to end-stage renal disease (ESRD), and the RAS is intimately involved in the pathogenesis of both. Thus, inhibition of angiotensin II (Ang II) formation with angiotensin-converting enzyme inhibitors (ACEIs) or Ang II action with ARBs effectively slows the progression of CKD by lowering BP and proteinuria. Although Ang II inhibition has been bedrock in CKD therapy for decades, ACEIs or ARBs do not completely prevent CKD progression. Evidence has been accumulating that AT_2R activation, either alone or combined with RAS blockade, has the potential to further slow CKD progression. This principle was triggered initially by studies demonstrating various potentially beneficial roles for the AT_2R in the kidney, including antiproliferative effects (Nakajima et al., 1995; Maric et al., 1998), apoptosis (Tanaka et al., 1995; Yamada et al., 1996), vasodilation (Siragy et al., 2000), inhibition of interstitial fibrosis (Morrissey and Klahr, 1999), and expression of the chemokine RANTES (Wolf et al., 1997). On the other hand, NF-κB, a key transcription factor in inflammatory disorders and stimulator of multiple chemokines, may be activated selectively by AT_2Rs (Lorenzo et al., 2002; Wolf et al., 2002).

2. Diabetic Nephropathy. The role of the AT_2R has been extensively studied in diabetic nephropathy (DN). Early studies demonstrated that streptozotocin

(STZ)-induced diabetes, a model of type 1, insulin-dependent diabetes, in the initial phase induced a significant reduction in renal AT_2R protein expression that was partially reversed by insulin treatment (Wehbi et al., 2001). Because AT_1R expression remained unchanged in diabetic versus control kidneys, an increase in AT_1R/AT_2R balance was inferred in the pathogenesis of DN. Subsequent studies on the effects of long-term STZ diabetes in SHR, however, showed that renal AT_1R and AT_2R expression levels (both mRNA and protein) were reduced in parallel (Bonnet et al., 2002). However, AT_2 R downregulation was not uniformly observed in the STZ-induced diabetic rat model, as others documented enhanced receptor expression in brush border and basolateral membranes of renal proximal tubule cells in this model (Hakam et al., 2006) and in human renal proximal tubule cells in response to high exogenous glucose exposure mediated by interferon regulatory factor-1 (Ali et al., 2010).

Substantial clarification of the role of AT_2R in DN emerged when the availability of genetic AT_2R-KO technology was applied to this disease. STZ-treated AT_2R-KO, when compared with wild-type control mice, displayed evidence of accelerated DN, including renal hypertrophy, tubule apoptosis, progressive extracellular matrix (ECM) formation, and increased glomerular filtration rate (GFR) (Chang et al., 2011). Renal oxidative stress (measured by heme-oxygenase-1 gene expression and ROS generation) and the expression of intrarenal RAS components such as angiotensinogen, AT_1Rs, and the ACE gene was augmented, whereas ACE2 gene expression was reduced in the renal proximal tubules (RPTs) of AT_2R-KO. These studies suggested that AT_2R deficiency accelerates the development of DN, which was mediated at least in part by increased oxidative stress and elevated ACE/ACE2 ratio in RPTs.

Further understanding of the role of AT_2Rs in DN was achieved with the availability of selective nonpeptide AT_2R agonist C21. In STZ-diabetic rats, C21 limited the usual increase in microalbuminuria, reduced renal interstitial inflammatory markers [tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and isoprostane] and increased NO and cyclic GMP (cGMP) formation without change in BP (Matavelli et al., 2015). Similarly, C21 significantly attenuated the diabetes-induced increase in cystatin C, albuminuria, mesangial cell expansion, and glomerulosclerosis in STZ-diabetic Apo E-KO mice (Koulis et al., 2015). Consistent with these findings, C21 inhibited the expression of proteins implicated in oxidative stress, inflammation, and fibrosis [p47phox, osteopontin, monocyte chemoattractant protein-1 (MCP-1), TNF-α, TGF-β, and CTGF] and increased gelatinases MMP-2 and MMP-9 in parallel with reduced extracellular matrix production (Koulis et al., 2015). Interestingly, KO mouse studies have shown that the receptor for advanced glycation end products (RAGE), which contributes to the development and progression of DN, may do so at least in part by inhibition of AT_2R expression (Sourris et al., 2010). In addition, the ACE2 activator diminazene aceturate delays DN by increasing both ACE2 and AT_2R expression in STZ type 1 DN (Goru et al., 2017). Taken altogether, the aforementioned studies suggest that AT_2R activation with C21 is protective against the development of type 1 DN.

The effects of AT_2R activation on DN also have been studied in experimental models of type 2 diabetes. In the Zucker diabetic fatty (ZDF) rat, a well recognized model of type 2 diabetes, C21 reduced glomerular, tubulointerstitial, and perivascular fibrosis, macrophage infiltration, and TNF-α expression and improved albuminuria, but a combination of C21 plus losartan was required to reduce albuminuria by the end of the study (Castoldi et al., 2014). In another type 2 diabetic model, Wistar rats fed a high-fat diet and administered low-dose STZ, pharmacologic blockade of AT_2Rs activated NF-κB signaling and increased ACE2 expression, the latter thought to be a compensatory response to the increased inflammatory mediators and oxidative stress (Pandey et al., 2015). The renal antiapoptotic and anti-inflammatory effects of telmisartan were significantly accentuated by C21 administration in this model, as indicated by reduced expression of several apoptotic markers and NF-κB–driven inflammatory molecules (Pandey and Gaikwad, 2017). The combination of C21 and telmisartan improved renal dysfunction, renal morphologic abnormalities, and hemodynamic disturbances in this type 2 DN model.

The role of AT_2Rs in the obese Zucker rat (OZR), a model of the metabolic syndrome, has also been extensively studied. The OZR develops spontaneous renal injury that is initiated by obesity associated with hyper-insulinemic prediabetes (Kurtz et al., 1989). The AT_2R agonist CGP42112A (CGP) reduced renal inflammatory and oxidative stress markers to levels observed in lean control rats (Sabuhi et al., 2011), and C21 decreased elevated levels of TNF-α and IL-6, whereas PD123319 lowered renal levels of the anti-inflammatory cytokine interleukin-10 (IL-10) (Dhande et al., 2013). In OZRs, C21 was also protective against high salt diet-induced proteinuric kidney injury (Patel et al., 2016). In summary, the accumulated evidence suggests that AT_2R activation with C21 is protective against DN in metabolic syndrome/prediabetes as well as types 1 and 2 diabetes.

3. Ischemic Nephropathy. Ischemic nephropathy describes the loss of renal function and/or renal parenchymal mass due to stenosis or occlusion of the renal artery and/or its branches. This condition is

commonly the result of atherosclerotic renal vascular disease, but other causes include fibromuscular dysplasia, arteritis, embolic disease, and aortic dissection. The RAS is intimately involved in the pathogenesis of ischemic nephropathy, wherein ischemia induces an early increase in intrarenal Ang II biosynthesis, AT_1R activation, vasoconstriction, and reduced glomerular filtration rate (GFR) (Kontogiannis and Burns, 1998). An early study indicated a protective role of AT_2Rs in the renal wrap (Grollman) model of ischemic kidney injury, wherein AT_2 R blockade with PD123319 prevented the hypotensive response to AT_1R blockade (Siragy and Carey, 1999). This was among the first studies suggesting that at least some of the beneficial actions of AT_1R blockade are transduced by AT_2R stimulation via the BK, NO, and cGMP signaling cascade. A more commonly employed experimental model of ischemic nephropathy is the 5/6 nephrectomy model, which undergoes Ang II-dependent induction of AT_2R expression in the ischemic kidney after ablation (Vázquez et al., 2005). Transgenic overexpression of AT_2Rs in vascular smooth muscle cells decreased urinary albumin excretion, glomerular size, and expression of platelet-derived growth factor-BB and TGF-β in 5/6 nephrectomy compared with wild-type mice (Hashimoto et al., 2004). These changes were due to AT_2R overexpression as they were abolished with PD123319. In this model, AT_1R blockade is associated with AT_2R upregulation, and AT_2R inhibition with PD123319 potentiates renal and vascular damage, suggesting that AT_2Rs represent a counterregulatory system preventing spread of tissue damage due to renal ablation. This idea was further supported by observations that renal ablation resulted in greater BP-independent impairment of renal function, glomerular injury, albuminuria, renal monocyte chemotactic protein-1, and RANTES expression as well as mortality in AT_2R-KO than wild-type mice (Benndorf et al., 2009). Subsequent studies in this model have confirmed that the beneficial effects of AT_1R blockade on kidney injury are complex and involve both inhibition of deleterious AT_1R actions and stimulation of beneficial AT_2R effects (Naito et al., 2010).

Another model of kidney ischemia is the ischemia-reperfusion model of renal injury and repair, which induces a transient increase plasma creatinine/urea nitrogen and proteinuria. C21 reversed these changes and increased the levels of IL-10 (Ali et al., 2021). Subsequently, in the course of reperfusion, C21 increased regulatory T and IL-10 producing cells and reduced kidney injury molecule-1 and neutrophil gelatinase-associated lipocalin, suggesting a role of AT_2Rs in kidney repair.

Renovascular hypertension also induces ischemic nephropathy accompanied by increased RAS activity and high BP. In an experimental model of this disease, 2-kidney, 1-clip Goldblatt hypertension, the development of inflammation and generation of ROS are characteristic of the ischemic kidney and are followed by progressive interstitial fibrosis. The early phase (4 days after clipping) was studied in this model (Matavelli et al., 2011); clipped kidneys were characterized by increased renal expression of AT_1Rs, AT_2Rs, TNF-α, IL-6, and TGF-β and reductions in renal interstitial fluid NO and cGMP levels. In contrast, C21 caused a significant reduction in renal TNF-α, IL-6, and TGF-β and increased NO and cGMP levels independent of BP. Thus, AT_2R activation reduced early renal inflammatory responses and improved NO and cGMP production in renovascular hypertension irrespective of BP. Further, NO production in response to AT_2R activation sustained renal oxygenation in the clipped kidney of early Goldblatt hypertensive rats (Palm et al., 2008). Because renal tissue hypoxia may be critical in the progression of CKD (Fine et al., 1998), the potential protective role of Ang II on tissue oxygenation via AT_2Rs is a compelling area for future study.

4. Focal Segmental Glomerulosclerosis. Focal segmental glomerulosclerosis (FSGS) is a cause of kidney disease, which is irreversibly progressive. Mesangial matrix expansion and basement membrane thickening are the initial structural changes leading to fibrosis, which is focal and segmental in the glomerulus (D'Agati et al., 2011). Pharmacological as well as genetic studies suggest that the AT_2R is renoprotective, including against structural changes in the glomerulus. In addition to amelioration of glomerular injury in the 5/6 nephrectomy model detailed above, pharmacological activation of AT_2Rs by C21 prevented focal segmental cellularity, mesangial expansion, and basement membrane thickening in obese Zucker rats fed high-sodium diet (Dhande et al., 2013; Patel et al., 2016). The protective effects of AT_2Rs were associated with attenuated proteinuria, a renal functional injury marker, in AT_2R-overexpressing mice and in animals treated with C21 (Hashimoto et al., 2004; Patel et al., 2016). A very recent study in mice where FSGS was induced by adriamycin treatment has provided further evidence for protective effects of AT_2Rs in this disease. FSGS was more severe in AT_2R-KO mice compared with wild-type FSGS mice, as evidenced by profound podocyte loss, glomerular fibrosis, and albuminuria; furthermore, C21 treatment of wild-type FSGS mice reduced podocyte injury (Liao et al., 2022).

5. Unilateral Ureteral Obstruction. Unilateral ureteral obstruction (UUO) is a well-characterized model of progressive renal tubulointerstitial fibrosis driven in large part by increased Ang II levels acting via AT_1Rs in the obstructed kidney (Sharma et al., 1993; Ishidoya et al., 1995). AT_2R blockade with PD123319 was found to exacerbate the increase in interstitial

volume and collagen IV matrix score of rats with a unilateral obstructed kidney (Morrissey and Klahr, 1999). These results were confirmed in AT_2R-KO, which displayed more severe renal interstitial fibrosis, collagen deposition, and fibroblast/myofibroblast infiltration as well as reduced apoptosis compared with wild-type controls (Ma et al., 1998). These studies demonstrate that the AT_2R significantly affects the remodeling process within the renal interstitial compartment, likely by regulating the population of collagen-producing cells. Studies also have shown that the antifibrotic actions of relaxin via its relaxin family peptide receptor both in UUO and in vitro are inhibited by PD123319 or when relaxin is administered to AT_2R-KO (Chow et al., 2014).

6. Hypertensive Nephropathy. Although the role of AT_2Rs in the pathophysiology of primary hypertension has been extensively evaluated, few studies are available on the specific role of AT_2R in hypertensive nephropathy. Stroke-prone spontaneously hypertensive rats (SHR-SP) constitute a model of spontaneous hypertension that develops renal inflammation and fibrosis (Gianella et al., 2007). Chronic C21 treatment dose-dependently delayed the appearance of proteinuria and reduced mortality in salt-loaded SHR-SP independent of BP (Gelosa et al., 2009). C21 further attenuated renal inflammation and fibrosis by reducing macrophage infiltration, neo-expression of vimentin and collagen deposition, beneficial effects that were abolished by PD123319.

7. Cyclosporin Nephropathy. Cyclosporin A is a calcineurin inhibitor widely used as an immunosuppressant in organ transplantation. However, one of the untoward effects of cyclosporine A administration is nephropathy characterized by tubule atrophy, inflammatory cell infiltration, and hyalinosis of the afferent arterioles followed by tubulointerstitial fibrosis. Chronic cyclosporin A nephropathy is a major cause of renal allograft dysfunction and failure in transplant recipients. Cyclosporin A administration to rats increased glomerular and predominantly tubulointerstitial fibrosis at 1 and 4 weeks. Chronic C21 administration (4 weeks) to rats with cyclosporine A nephropathy reversed inflammatory cell infiltration and tubulointerstitial fibrosis to pretreatment control levels independently of BP (Castoldi et al., 2016).

8. Sickle Cell Nephropathy. Sickle cell nephropathy, a leading cause of mortality in patients with sickle cell anemia, comprises a number of renal pathologies, including both glomerular and tubular defects. A prominent tubular defect is impaired urine concentrating ability secondary to erythrocyte sickling-induced *vasa recta* occlusion with ischemia leading to loss of normal medullary osmotic gradient. AT_2R-KO have reduced urine osmolality compared with control wild-type animals, and administration of AT_2R agonist C21 in the presence of AT_1R blockade with losartan improved urine osmolality to control wild-type levels (Roy et al., 2018). Improvement in urine concentrating ability with AT_2R activation demonstrates another potentially important renoprotective role of AT_2Rs in nephropathy.

9. Disorders of Na^+ and Fluid Retention. Renal AT_2 acting cooperatively with dopamine receptors, counterbalance Na^+ retention elicited by Ang II via AT_1Rs (see Section V.A). AT_2R activation accounts for natriuresis in response to AT_1R blocker therapy and maintains normal Na^+ balance in Ang II-dependent hypertension, reducing BP. Since AT_2Rs induce natriuresis in the RPT, a nephron site for which no natriuretic/diuretic therapy is currently available, AT_2R agonists are promising candidates for treatment of disorders of Na^+/fluid retention such as CKD, heart failure, and Na^+-sensitive/volume-dependent hypertension.

10. Summary. Substantial evidence from experimental animal studies underscores the protective role of the AT_2R in DN (prediabetes and type I and type II diabetes), ischemic nephropathy, UUO, hypertensive nephropathy, and toxic (cyclosporine) nephropathy. Beneficial AT_2R actions in CKD include inhibition of renal inflammation and ROS generation as well as the delay or prevention of renal fibrosis, the foundation of CKD progression to end-stage renal disease (ESRD). Figure 11 provides a schematic representation of the signaling pathways that underlie beneficial actions of AT_2R agonism in CKD. Although many of the animal studies cited here are acute and more long-term information is needed, the available observations, in aggregate, encourage early phase clinical testing of nonpeptide AT_2R agonist therapy, alone or combined with an ARB, in human CKD.

B. Heart

1. Acute Myocardial Infarction. Cardiac AT_2R expression changes post–MI in a time-dependent manner. In adult rats, AT_2Rs were expressed in about 10% of cardiomyocytes, and this proportion did not change within the first day after MI (Busche et al., 2000). However, 1 week after MI, cardiac expression of the AT_2R in rats was enhanced (Nio et al., 1995; Zhu et al., 2000), whereas in the intermediate phase, 4 weeks post-MI, expression levels of the AT_2R were decreased (Lax et al., 2004). In humans, AT_2R expression was reported to be stronger in infarcted than in adjacent noninfarcted myocardium (Wharton et al., 1998).

The first studies addressing the role of the AT_2R in MI were performed in genetically altered mice, either AT_2R deficient or overexpressing. For instance, MI induced by coronary artery ligation in male and female mice with targeted AT_2R gene deletion caused cardiac rupture (Ichihara et al., 2002), exaggerated early left ventricular dilation and cardiac dysfunction, and increased mortality, thus supporting a protective effect

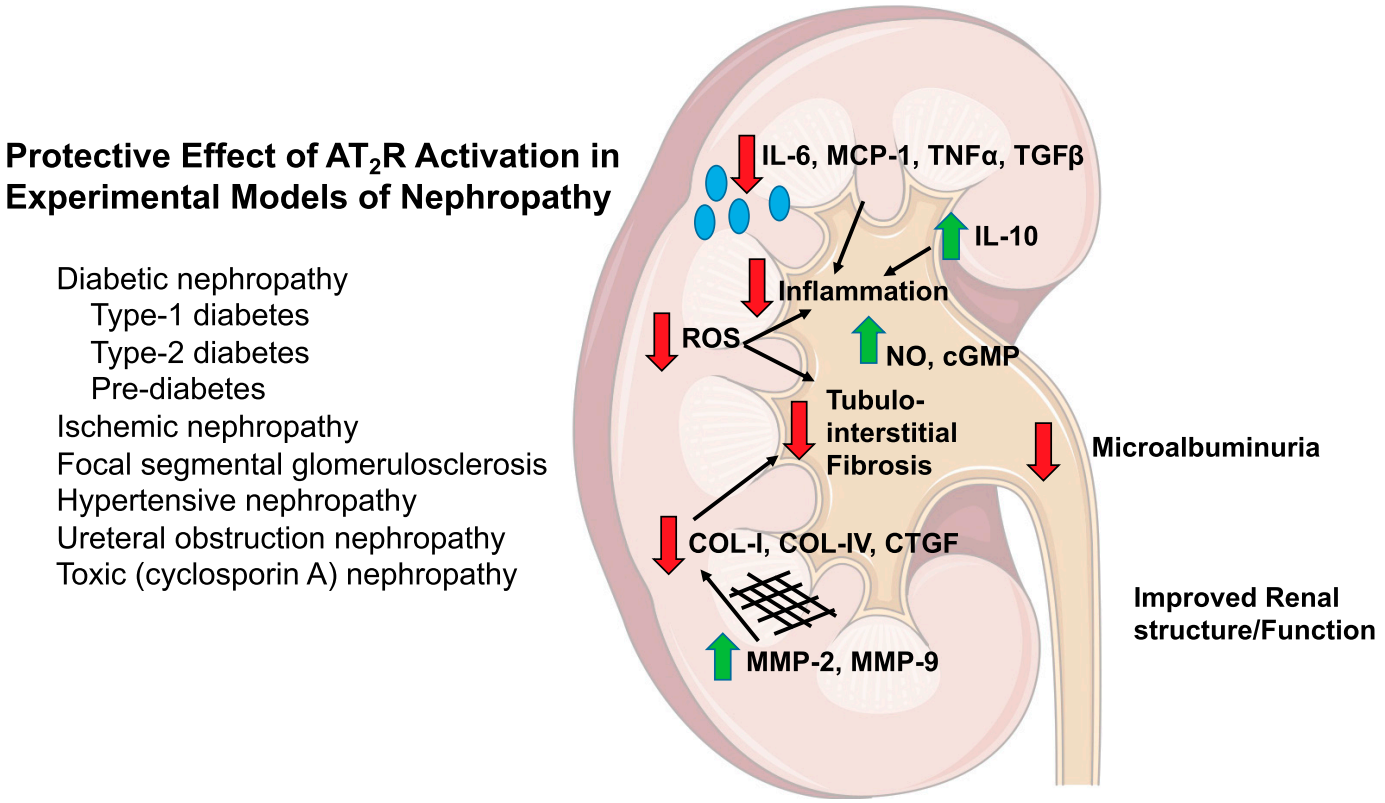

Fig. 11. Schematic representation of selected signaling pathways whereby AT_2R activation conveys renal protective effects in various experimental models of nephropathy. AT_2R activation decreases (red arrow) or increases (green arrow) various factors/processes. COL-I, collagen I; COL-IV, collagen-IV; MCP-1, monocyte chemoattractant protein-1; ROS, reactive oxygen species.

of the AT_2R in MI (Adachi et al., 2003; Oishi et al., 2003). In a model of myocardial cryoinjury, AT_2R deficiency resulted in a prohypertrophic effect, suggesting an antihypertrophic action of the AT_2R (Brede et al., 2003). Similar to AT_2R-KO, AT_2R blockade with PD123319 further impaired cardiac performance after MI (Kuizinga et al., 1998). The reverse approach (i.e., cardiac AT_2R overexpression in mice) led to improved contractile function as shown by echocardiography or cardiac magnetic resonance (CMR) imaging (Yang et al., 2002; Bove et al., 2004; Isbell et al., 2007; Qi et al., 2012; Xu et al., 2014). Specifically, contractility of peri-infarcted myocardium was improved in these mice as shown by CMR tagging (Bove et al., 2005). AT_2R overexpression and $AT1_aR$-KO had additive effects, which at least in part were thought to be due to the significantly lower BP compared with AT_2R-overexpressing mice without $AT1_aR$-KO (Voros et al., 2006). However, the additive effect was not apparent in AT_2R-overexpressing mice treated with losartan and with a comparable lowering of BP. Several studies reported an improvement of cardiac function after MI by AT_1R blockade (Jalowy et al., 1998; Xu et al., 2000, 2002; Jugdutt et al., 2001; Jugdutt and Menon, 2004; Oishi et al., 2006) and provided evidence that indirect AT_2R stimulation was involved in these cardioprotective effects. Direct AT_2R stimulation with C21 in Wistar rats with left coronary ligation improved systolic and diastolic function coinciding with a smaller infarct scar (Kaschina et al., 2008). Anti-inflammation (by reduced cytokine expression) and antiapoptosis (by rescue of p38 and p44/42 MAPK activity) were identified in this study as AT_2R-mediated tissue-protective mechanisms. Improvement of early ventricular remodeling, in particular prevention of early ventricular dilatation, hypertrophy, or rupture, are further proposed AT_2R effects (Ichihara et al., 2002; Brede et al., 2003; Oishi et al., 2006; Qi et al., 2012). Generally, knowledge about AT_2R-coupled signaling in MI models is still rather fragmented. Although evidence for an involvement of NO signaling is consistent (Brede et al., 2003; Bove et al., 2004), a role for BK acting through the B_2R is controversial with one study in support (Jalowy et al., 1998) and one contradicting this mechanism of action (Isbell et al., 2007). Involvement of prostaglandins may be another mechanism (Jalowy et al., 1998; Ichihara et al., 2002) as is AT_2R-induced activation of the Na^+-HCO_3 symporter (NBC) in the infarcted myocardium and consequent prevention of intracellular acidosis (Sandmann et al., 2001).

Interestingly, populations of AT_2R-expressing CD8 (CD8+/AT_2R+) and CD4 (CD4+/AT_2R+) T cells as well as stem cells (c-kit+/AT_2+) have been described

in the peri-infarct tissue (Altarche-Xifró et al., 2009; Curato et al., 2010; Skorska et al., 2015). Unlike their non–AT_2R-expressing counterparts, these cells had anti-inflammatory and regenerative properties. Infusion of such CD8+/AT_2R+ and CD4+/AT_2R+ T cells (Curato et al., 2010; Skorska et al., 2015) or of bone marrow mononuclear cells, which were preconditioned by AT_2R stimulation (Xu et al., 2013), reduced infarct size and improved cardiac function.

Although the overwhelming majority of studies supported a beneficial effect of the AT_2R in MI, there are also two studies that did not report any impact of the AT_2R on post-MI cardiac performance: one performed in AT_2R-deficient mice (Tschöpe et al., 2005), and the other used treatment with C21 at a dose (0.3 mg/kg per day by minipump), which is standard for intraperitoneal (i.p.) bolus administration but due to the rather short half-life of C21 is likely too low to reach efficient plasma concentrations when continuously applied as minipump infusion (Jehle et al., 2012; Kaschina et al., 2014).

Finally, further evidence for a protective role of the AT_2R in MI came from the identification of an association of an AT_2R polymorphism (−1332 G/A) and premature coronary artery disease in males (Alfakih et al., 2005).

2. Heart Failure. Chronic heart failure (HF) is a common complication of MI, which is why long-term follow-up MI models are frequently used for preclinical HF studies. Cardiac AT_2R overexpression (Qi et al., 2012), indirect AT_2R simulation by AT_1R-blockade (Liu et al., 1997; Thai et al., 2003), or direct stimulation of peripheral (Lauer et al., 2014) or central AT_2Rs (Gao et al., 2011) has been investigated in such models. In addition, C21 with or without additional AT_1R blockade has been tested in a model of HF caused by adrenergic overstimulation with isoproterenol (Ulutas et al., 2021). All of the above-cited studies found an AT_2R-mediated improvement of cardiac function, and most of them reported improved cardiac remodeling, in particular a reduction of cardiac fibrosis, which was associated with a dramatic inhibition of TGF-β1 expression and an amelioration of the disbalanced MMP-9/TIMP1 (tissue inhibitor of metalloproteinase 1) ratio (Liu et al., 1997; Qi et al., 2012; Lauer et al., 2014). An additional, protective mechanism was an increase in NO bioavailability, resulting in rescue of the impaired, post-MI vasorelaxant response (Thai et al., 2003). The detrimental, increased sympathetic outflow in HF was inhibited by centrally applied C21 (Gao et al., 2011), which is a promising mechanism, but with limited translational potential unless a suitable route of administration for delivery of an AT_2R agonist into the brain can be found (e.g., nose-to-brain application) (see Section VI.F.1).

The reader is referred to a number of review articles for more detail (Widdop et al., 2003; Carey, 2005; Kaschina et al., 2014, 2017).

3. Cardiac Hypertrophy. Unlike MI-induced cardiac remodeling, in which AT_2R effects are generally cardioprotective, there have been many conflicting studies concerning the involvement of AT_2Rs in generalized cardiac hypertrophy. Indeed, either pro- or antihypertrophic effects of AT_2Rs were noted, derived from initial AT_2R-KO studies whereby left ventricular hypertrophy (LVH) was induced by aortic banding, Ang II infusion, or L-NAME; the effects within each study paralleled changes in cardiac fibrosis (Widdop et al., 2003; Gross et al., 2004). Additionally, in mice with cardiac-specific AT_2R overexpression, cardiac hypertrophy induced by Ang II was unaffected (Sugino et al., 2001; Kurisu et al., 2003), whereas aortic banding-induced LVH was inhibited (Yan et al., 2008). In rats, early postnatal cardiac AT_2R gene delivery inhibited cardiac mass in adult SHR (Metcalfe et al., 2004) as well as Ang II-induced cardiac hypertrophy in adult rats (Falcón et al., 2004). Thus, there is no clear consensus from AT_2R gene modification studies for AT_2Rs to influence cardiac hypertrophy since prohypertrophic (Senbonmatsu et al., 2000; Ichihara et al., 2001), antihypertrophic (Falcón et al., 2004; Gross et al., 2004; Metcalfe et al., 2004; Yan et al., 2008) and neutral (Akishita et al., 2000b; Sugino et al., 2001; Wu et al., 2002; Kurisu et al., 2003) effects of AT_2Rs on cardiac mass have been reported [see also Jones et al. (2008) and Steckelings et al. (2010)] . Inconsistent findings for Ang II-induced cardiac hypertrophy have also been reported in vitro using AT_2R-transfected cardiomyocytes (D'Amore et al., 2005; Aránguiz-Urroz et al., 2009). All of the 'antihypertrophic' as well as 'neutral' studies for cardiac hypertrophy, listed above, also exhibited cardiac antifibrotic effects (see Section VI.B.3/4), which points to a greater AT_2R sensitivity for protection against extracellular matrix deposition than cardiomyocyte hypertrophy, at least with AT_2R gene manipulation. Similarly, the ability of PD123319 to reverse the cardioprotective effects of AT_1R blockade, indirectly implicating AT_2R involvement, was apparent for antifibrotic but not antihypertrophic cardiac effects in SHR (Varagic et al., 2001; Jones et al., 2012). More recently, there have been studies that directly addressed AT_2R-induced modulation of cardiac mass using AT_2R agonists, from which there is greater consensus for antihypertrophic effects in the heart. Rehman et al. (2012) demonstrated cardioprotective effects of C21 in SHR-SP by a reduction in molecular markers of cardiac hypertrophy although LVH per se was not reduced. In other models in which there was no evidence of increased baseline cardiac hypertrophy such as diabetic Zucker rats (Castoldi et al., 2019)

and high salt-fed rats (Dopona et al., 2019), C21 reduced cardiomyocyte size on histologic analysis but not gross cardiac hypertrophy. In Ang II-infused rats, daily intraperitoneal injections of C21 prevented LVH (Castoldi et al., 2021). CGP42112A did not modify cardiac mass in high salt-fed FVB/N mice (Wang Y et al., 2020), which was not surprising given the lack of cardiac hypertrophy in this model, whereas NP-6A4 reduced cardiac hypertrophy in Zucker rats (Gavini et al., 2021). Collectively, these antihypertrophic effects due to AT_2R stimulation occurred independently of BP in both normotensive and hypertensive models that exhibited cardiac hypertrophy.

4. Hypertension-Induced Cardiac Fibrosis. As described in earlier sections (Sections III.E.2 and VI.B.1), the AT_2R is well represented across a variety of cell types in the heart and, importantly, is highly expressed in cardiac fibroblasts in diseased human hearts (Brink et al., 1996; Tsutsumi et al., 1998; Wharton et al., 1998; Widdop et al., 2003), which is a key cellular source of ECM proteins that play a pivotal role in cardiac remodeling. The majority of cardiac studies that have investigated the role of AT_2Rs in cardiac fibrosis have used models of cardiac injury such as acute MI (see Section VI.B.1). In contrast, there have been few reports that directly inform on AT_2R involvement in hypertensive states. In MI-induced HF, there is a reparative response to acute ischemic injury since the latter induces cardiomyocyte death. Under these circumstances, there is an acute inflammatory phase to remove necrotic tissue followed by increased collagen production to stabilize cardiac structural integrity at the site of MI injury, although this profibrotic activity often spills into regions remote from the initial injury (Prabhu and Frangogiannis, 2016; Wang et al., 2022). In contrast, in hypertensive heart disease with no obvious acute injury, there is persistent inflammation and oxidative stress that leads to ECM buildup throughout the heart (Berk et al., 2007). This is exemplified in various reactive cardiac fibrosis models, as recently reviewed (Wang et al., 2022). This so-called reactive fibrosis does not usually have a defining trigger and is a common sequela in hypertension and cardiometabolic disease. The contrasting effects of AT_2R stimulation on ECM turnover after acute MI- or hypertension-induced cardiac fibrosis have been reviewed elsewhere (Wang Y et al., 2017). In early studies, Varagic and colleagues (2001) reported that PD123319 could reverse candesartan-induced reductions in left and right ventricular fibrosis in 22-week-old male SHR over a 12-week treatment regimen. In contrast, Jones et al. (2012) studied both adult (20 weeks) and aged (20 months) male SHR and found that although candesartan did not reduce cardiac fibrosis over 4 weeks, it reduced perivascular fibrosis, which was reversed by PD123319 in aged SHR. Collectively, these data are consistent with an antifibrotic role of the AT_2R in hypertensive hearts, at least as part of the therapeutic effect of ARBs that was independent of any BP effects.

Surprisingly, there are relatively few reports on the effects of AT_2R stimulation on hypertension-related end organ damage, at least in the heart. C21 was given chronically to SHR-SP that had markedly elevated BP (Rehman et al., 2012). In this study, C21 reduced myocardial interstitial as well as perivascular collagen content, and this cardiac antifibrotic effect was enhanced in combination with losartan. Ang II-induced hypertension also causes cardiac fibrosis. Initially, there were contrasting effects reported using different AT_2R-KO mice derived from different background strains, which has been discussed in detail previously (Widdop et al., 2003). However, an antifibrotic effect of AT_2R stimulation is generally accepted based on AT_2R-KO studies in which cardiac fibrosis was induced by either Ang II (Jones et al., 2008) or L-NAME (Gross et al., 2004). Consistent with this concept, Ang II-induced interstitial and perivascular fibrosis was blunted in mice in which cardiomyocytes overexpressed AT_2Rs (Kurisu et al., 2003). This effect was mediated via a BK/NO-dependent mechanism and was independent of BP, which was raised by Ang II in this model. Similarly, an antifibrotic effect was observed in rat hearts transduced 5 days after birth with lentivirus containing AT_2Rs, with rats then subjected to Ang II at 15 weeks of age (Falcón et al., 2004). Consistent with these findings, it was recently reported that, in Ang II-infused rats, C21 (0.3 mg/kg per day by i.p. injection for 1 or 4 weeks) prevented cardiac fibrosis (Castoldi et al., 2021).

The ingestion of high salt in the diet is another example of a cardiac fibrotic model, although high salt exerts a variable effect on BP (Wang et al., 2022). In male Wistar rats fed a high-salt diet (8% NaCl), there was a marked hypertensive effect accompanied by modest cardiac fibrosis in the left, but not right, ventricle. C21 was given by daily gavage commencing 4 weeks after the start of the high-salt diet as an intervention protocol for a further 11 weeks. C21 slightly reduced the markedly elevated BP while reducing cardiac fibrosis in both ventricles (Dopona et al., 2019). This antifibrotic effect is likely to be a class effect since the AT_2R agonists CGP42112A (Wang Y et al., 2020) and NP-6A4 (Gavini et al., 2021) also evoked cardiac antifibrotic effects in models without any background hypertension. Wang Y et al. (2020) reported that CGP42112A reversed high-salt (5%)–induced interstitial fibrosis and inhibited the high-salt–induced elevation in tissue inhibitor of matrix metalloproteinases-1 in male FVB/N mice over a 4-week treatment period in which candesartan cilexetil was ineffective. In addition, daily subcutaneous injections of NP-6A4 for a fortnight reduced cardiac fibrosis

and improved diastolic dysfunction in obese male Zucker rats (Gavini et al., 2021).

C. Blood Vessels

1. Vascular Remodeling. Remodeling of blood vessels occurs in response to injury, aging, or to various diseases associated with disturbances of blood flow, dysregulation of plasma lipid and glucose levels or with systemic inflammation. It can manifest as hyperplasia of intima (involving vascular endothelial cells) or media [involving vascular smooth muscle cells (VSMCs)], fibrosis (involving vascular fibroblasts), or calcification (van Varik et al., 2012; Renna et al., 2013). Victor Dzau's group investigated the role of the AT_2R in neointima formation in a series of studies using various genetic models. In the first of these studies, neointima proliferation was induced by balloon injury in rat carotid arteries (Nakajima et al., 1995). AT_2R overexpression in the injured area by viral transduction reduced proliferation of neointimal cells and thereby neointimal area by 70% compared with arteries treated with a control vector. This effect could be blocked by PD1232319 (Nakajima et al., 1995). A follow up study in AT_2R-KO or wild-type mice used a model of neointima proliferation induced by vascular inflammation triggered by placement of a polyethylene cuff around the femoral artery (Akishita et al., 2000a). Neointimal area and VSMC proliferation were increased by almost 100% by AT_2R deficiency, again indicating an inhibitory role of the AT_2R on neointima formation. In two further studies, the Dzau group showed that the AT_2R-mediated protection from neointima formation was sex and age dependent because the protective effect was stronger in females (Okumura et al., 2005) but weaker in aged animals (Okumura et al., 2011). Enhanced neointima formation in AT_2R-KO compared with wild-type mice was confirmed by an independent group in a model of neointima proliferation induced by wire injury of femoral arteries (Yamamoto et al., 2008). Vascular inflammation and neointima formation in the polyethylene cuff model were also inhibited by AT_1R blockade with valsartan, and this effect included indirect AT_2R stimulation because it was markedly attenuated by coadministration of PD123319 (Wu et al., 2001). A similar observation was made for hypertension-induced vascular hypertrophy, which is primarily driven by an increase in media thickness. Reduction of media-to-lumen ratio by the AT_1R-antagonist candesartan cilexetil in aged Wistar rats was reversed by concomitant AT_2R blockade with PD123319, suggesting again that the favorable effect of AT_1R-blockade was primarily due to indirect AT_2R stimulation (Jones et al., 2004). Of note, although AT_1R-blockade significantly lowered BP, which likely contributed to the antihypertrophic effect, AT_2R blockade was BP neutral, thus pointing to BP-independent effects of the AT_2R on vascular hypertrophy (Jones et al., 2004). This assumption is supported by two more studies: one reporting spontaneous media hypertrophy in femoral arteries of AT_2R-KO compared with wild-type mice, whereas there were no differences in BP between the two strains (Brede et al., 2001), and another reporting a greater wall-to-lumen ratio in coronary arteries from AT_2R-KO than from wildtype mice after aortic banding despite a comparable increase in BP (Akishita et al., 2000b).

In contrast to the above studies, which took an indirect approach and investigated the impact of AT_2R antagonism or knockdown on neointima proliferation, another study took the direct approach and treated mice with C21 after polyethylene cuff-induced vascular injury (Kukida et al., 2016). C21 treatment inhibited neointimal cell proliferation, neointima area, and expression of various inflammatory markers through a signaling cascade involving ATIP and PPARγ activation (Kukida et al., 2016). A combination of C21 with rosuvastatin in this model resulted in synergistic effects (Bai et al., 2016). Furthermore, in a model of balloon injury in rat carotid artery, perivascular infusion of CGP42112A (at that time thought to be an antagonist) significantly prevented neointima formation (Janiak et al., 1992). Details of the inflammatory mediators involved in AT_2 R-induced modulation of neointimal formation is noted in Section VI.C.3.

Two studies with similar design were published back-to-back in 2012 and examined the effect of AT_2R stimulation on fibrotic aspects of hypertension-induced vascular remodeling. One study used SHR-SP (Rehman et al., 2012), the other Wistar rats treated with the eNOS-inhibitor L-NAME (Paulis et al., 2012). In both studies oral treatment with C21 resulted in an attenuation of vascular stiffness and of extracellular matrix deposition. Both studies compared the effect of C21 with the effect of an AT_1R-blocker and unequivocally found that effects on vascular fibrosis and stiffening were similar, although only the AT_1R-antagonists lowered BP. For most measured parameters, there was no additive or synergistic effect of combined AT_1R blockade/AT_2R stimulation (Paulis et al., 2012; Rehman et al., 2012).

Vascular fibrosis due to hyperglycemia or hyperlipidemia also responded to AT_2R agonist treatment, which resulted in a reduction in collagen deposition and fibrotic markers (e.g., TGF-β, MMP activity) as shown in a model of high-fat diet–induced obesity (González-Blázquez et al., 2021a) and in Apolipoprotein E $(ApoE)^{-/-}$ mice with STZ-induced type I diabetes (Chow et al., 2016).

The latest addition to studies on AT_2R-mediated effects on vascular remodeling reported an attenuation of vascular calcification, which was induced by an

adenine/high-phosphate diet, in mice with VSMC-specific AT_2R overexpression and in Wistar rats treated with C21 (Kukida et al., 2019). Reduced phosphate-induced calcification or calcium deposition, respectively, were further found in rings from thoracic aorta and in primary VSMCs derived from AT_2R-overexpressing mice but not in aortic rings or cells from wild-type mice. These in vivo and in vitro effects could be blocked with the PPARγ-inhibitor GW9662, suggesting that this AT_2R effect involved PPARγ activation (Kukida et al., 2019).

Despite a vast majority of publications supporting a beneficial role of the AT_2R in vascular remodeling, there are also studies that opposed this view and reported no effect or even a pathology-promoting action of AT_2R in this context (Levy et al., 1996; Sabri et al., 1997; Otsuka et al., 1998; Cao et al., 1999; Nasser et al., 2014). However, all of these studies used the AT_2R antagonist PD123319, but not an agonist, for detection of AT_2R-mediated effects, which due to the potential partial agonistic property of PD123319 (see Section IV.C) may have led to false conclusions.

Notably, AT_2R stimulation has not only been reported to counteract mechanisms that mediate pathological, inward-hypertrophic vascular remodeling, but it also promotes outward vascular remodeling, which results in an increase in lumen diameter. This effect was described in a model of ligation of selected mouse mesenteric arteries, which induced high flow in the nonligated arteries. The authors showed that the increase in diameter (as a result of outward remodeling) of the high-flow arteries 7 days after ligation was dependent on AT_2R-mediated interleukin-17 production by T lymphocytes (Caillon et al., 2016).

2. Atherosclerosis. Ang II is well known to promote the pathogenesis of atherosclerosis via AT_1R activation (Daugherty and Cassis, 2004). In contrast, the effects of AT_2Rs on atherosclerosis are more controversial and dependent on the research tools being employed in each study. The AT_2R antagonist PD123319 potentiated Ang II-induced atherosclerosis in young (2-month-old) but not in aged (11-month-old) female ApoE-deficient mice from the same study (Daugherty et al., 2001). PD123319 was without effect in male Ang II-infused hypercholesterolemic low-density lipoprotein (LDL)-deficient mice (Daugherty et al., 2013) or male Ang II-infused ApoE-deficient mice (Johansson et al., 2005), whereas it was reported to reduce atherosclerosis in male diabetic ApoE-deficient mice (Koıtka et al., 2010). Although early studies that deleted the AT_2R inferred atheroprotective effects due to AT_2R activation, such findings were not universal. Exaggerated atherosclerotic lesions occurred when the AT_2R was deleted from male hypercholesterolemic ApoE-deficient mice (Iwai et al., 2005) or when AT_2R deficiency prevented a decrease in lesional macrophages, lipids, and collagen, which occurred in wild-type mice over time (Sales et al., 2005). In conrast, AT_2R deletion had not impact on atherosclerosis at all in hypercholesterolemic LDL-deficient mice (Daugherty et al., 2004), male Ang II-infused ApoE-deficient mice (Johansson et al., 2005) or male Ang II-infused hypercholesterolemic LDL-deficient mice (Daugherty et al., 2013). One study in male diabetic AT_2R- and ApoE-deficient mice even reported protection from atherosclerosis by AT_2R-KO (Koıtka et al., 2010). In contrast, consistent with an antiatherosclerotic effect mediated by the AT_2R, systemic overexpression of AT_2Rs in hypercholesterolemic LDL-deficient mice (Dandapat et al., 2008; Hu et al., 2008) or overexpression of vascular AT_2Rs in Ang II-infused ApoE-deficient mice (Takata et al., 2015) resulted in reduced vascular inflammation and oxidative stress together with reduced atherosclerotic lesion development. Of note, all of the aforementioned studies not only employed different experimental models but also differed on various aspects, including mode of facilitating atherosclerosis, gender, age of mice, duration of treatment, or study. Therefore, it is hardly surprising that such experimental differences led to variable outcomes.

Nevertheless, AT_2Rs are localized in plaque and inflammatory cells within plaque in both animal studies (Johansson et al., 2005; Sales et al., 2005; Zulli et al., 2006) and human atherosclerotic lesions (Johansson et al., 2008). Indeed, chronic treatment with CGP42112A over the final 4 weeks of a 16-week high-fat diet regimen in male ApoE-deficient mice resulted in reduced atherosclerotic lesions and improved NO bioavailability that was manifest by improved endothelial function in a PD123319-sensitive manner (Tesanovic et al., 2010; Kljajic et al., 2013) In analogous protocols, Ang-(1-7) also evoked atheroprotective effects that were partially AT_2R-mediated (Tesanovic et al., 2010). Ang II itself was also reported to be atheroprotective via AT_2Rs in hypercholesterolemic ApoE-AT_1R double KO mice since Ang II decreased plaque formation, whereas PD123319 increased plaque formation in the same model (Tiyerili et al., 2012). Finally, C21 was recently reported to exert atheroprotective effects along the entire length of the aorta in diabetic ApoE-deficient mice (Chow et al., 2016) using an identical model in which, in contrast to this finding, PD123319 was reported previously to reduce lesion burden (Koıtka et al., 2010). Based on the weight of evidence from AT_2R overexpression and direct AT_2R stimulation studies, the AT_2R can be considered a therapeutic target for atherosclerosis, which most likely involves an anti-inflammatory component (see next section).

3. Vascular Inflammation. There is much evidence to suggest that AT_2Rs mediate an anti-inflammatory profile, particularly in cell-based studies (Rompe et al., 2010a,b; Patel et al., 2020; Fatima et al., 2021) and in the kidney (Sabuhi et al., 2011; Dhande et al., 2013), where AT_2R stimulation reduced proinflammatory cytokines such as IL-6 and increased the anti-inflammatory cytokine IL-10 (see Section VI.A). Consistent with the majority of aforementioned studies reporting on AT_2R-mediated antiatherosclerotic effects (see Section VI.C.2), there were also reductions in elevated inflammatory cytokines and oxidative stress in the vessel wall and/or plaque lesion evoked by AT_2R stimulation (Iwai et al., 2005; Sales et al., 2005) or AT_2R overexpression (Dandapat et al., 2008; Hu et al., 2008; Takata et al., 2015). Along similar lines, AT_2R stimulation increased NO bioavailability in aortic tissue from hypercholesterolemic ApoE-deficient mice after chronic treatment with CGP42112A (Kljajic et al., 2013), Ang-(1-7) (Tesanovic et al., 2010), or Ang IV (Vinh et al., 2008a,b), which is likely to limit oxidative stress and contribute to vascular anti-inflammatory effects of such peptides.

Distinct from atherosclerotic studies, there are also a number of studies that have examined the influence of AT_2Rs on vascular injury. Balloon injury to rat carotid arteries led to detectable AT_2R mRNA in the vessel wall, which was not detected in uninjured vessels (Nakajima et al., 1995). Moreover, AT_2R transfection into this balloon-injury model led to attenuation of the usual neointimal formation due to the balloon injury (Nakajima et al., 1995), linking increased vascular AT_2Rs with protective effects in vasculature. The introduction of a polyethylene cuff around the femoral artery in mice has also been used as a model to induce inflammatory responses with enhanced proliferation and migration of VSMCs from media into the intima. Femoral cuff placement caused a neointimal lesion with increased expression of proinflammatory cytokines, including interleukin-1β (IL-1β), IL-6, and TNF-α together with infiltration of leukocytes and macrophages, effects that were enhanced in AT_2R-KO (Akishita et al., 2000a; Wu et al., 2001; Okumura et al., 2005). The AT_2R antagonist PD123319 also increased neointimal formation in the cuff-induced vascular injury model (Akishita et al., 2000a). This cuff model of nonocclusive vascular injury was associated with increased AT_2R expression in the injured vessel in the aforementioned studies and has been used to identify that neointimal formation and elevated chemokine expression and superoxide production in injured femoral arteries were all attenuated in female mice compared with male counterparts, which was associated with an estrogen-sensitive upregulation of AT_2Rs (Okumura et al., 2005, 2011). In the same model, C21 decreased neointimal formation and expression of MCP-1, TNF-α, IL-1β, and oxidative stress (Kukida et al., 2016; Nakaoka et al., 2016). These anti-inflammatory effects of C21 were in part mediated by PPARγ (Kukida et al., 2016) or were synergistic with rosuvastatin on preventing vascular remodeling (Bai et al., 2016). Interestingly, the ability of valsartan to inhibit neointimal inflammation was greater in female than male mice, which was diminished in AT_2R-KO (Wu et al., 2001; Okumura et al., 2005), implying an AT_2R component to the anti-inflammatory effect of AT_1R blockade.

The vascular anti-inflammatory effect of AT_2R stimulation has also been examined in hypertensive animals chronically treated with C21. For example, in salt-loaded SHR-SP, the severe vascular lesions in the kidney, exemplified by hyperproliferative and hypertrophic arterioles (and marked tubulointerstitial fibrosis), were all prevented by relatively high C21 doses (Gelosa et al., 2009). In the same animal model but without salt loading, C21 prevented superoxide production in aortic and mesenteric vessels as well as aortic vascular cell adhesion molecule 1 (VCAM-1) expression and monocyte/macrophage infiltration in aorta and kidney (Rehman et al., 2012).

4. Hypertension. The AT_2R is thought to inhibit AT_1R-mediated contraction, and there are many instances in which reduced Ang II-induced pressor activity in rodents likely reflects tonic AT_2R-mediated relaxation. For example, manipulation of AT_2R levels has direct effects on BP or vessel phenotype. AT_2R-KO mice exhibited either no change (Hein et al., 1995a) or increased (Ichiki et al., 1995) basal BP, whereas both research groups reported increased sensitivity to Ang II-evoked pressor activity, reflecting an inhibitory effect of AT_2Rs on vascular tone. Similarly, vascular AT_2R overexpression, although not affecting basal BP, virtually abolished Ang II-mediated pressor responses in vivo and reduced aortic contraction in vitro (Tsutsumi et al., 1999). Intriguingly, these protective vascular effects of the AT_2R were manifest despite the maintained predominance of vascular AT_1Rs in the aforementioned study (AT_1R:AT_2R ratio of $\sim$10:1 in wild-type aorta vs. $\sim$3:1 in overexpressed AT_2R in aorta). This finding emphasizes the vasoprotective potential of the AT_2R even when its own (increased) expression was still at lower relative levels than the AT_1R. Abdominal aortic banding evokes hypertension and markedly increases thoracic aortic AT_2R expression in rats (Yayama et al., 2004) and mice (Hiyoshi et al., 2004; Yayama et al., 2006), as also seen in 2-kidney-1-clip-hypertensive mice (Hiyoshi et al., 2005). At the same time, Ang II-mediated aortic contraction was reduced in these mice probably due to AT_2R upregulation as indicted by PD123319-sensitivity and eNOS-cGMP involvement in this effect (Hiyoshi et al., 2004, 2005; Yayama et al., 2004, 2006). Given the widespread but low AT_2

R expression in vasculature, coupled with demonstrations of in vitro vasorelaxation (see vascular Sections III.E.3 and V.C), it is not surprising that selective stimulation of AT_2Rs by CGP42112A (infused subcutaneously) lowered BP, provided that there was a background of AT_1R blockade in conscious rats (Barber et al., 1999; Carey et al., 2001). Indeed, a number of AT_2R agonists (all infused subcutaneously), including CGP42112A (Barber et al., 1999), Ang-(1-7) (Walters et al., 2005), C21 (Bosnyak et al., 2010; Brouwers et al., 2013), β-Ile^5-Ang II (Jones et al., 2011), and β-Pro^7-Ang III (Del Borgo et al., 2015) all acutely reduced BP in conscious SHR due to reduced vascular tone in hypertensive vessels since these acute antihypertensive effects were associated with increased peripheral vasodilation (Li and Widdop, 2004).

However, the majority of studies using oral administration of C21 ranging from 4 days to 6 weeks failed to reduce BP in various hypertensive models (Gelosa et al., 2009; Matavelli et al., 2011; Paulis et al., 2012; Rehman et al., 2012), although it did so in high salt-fed obese Zucker rats (Ali et al., 2015). In addition, renal interstitial infusion of C21 reduced BP in Ang II-induced hypertension over 7 days in rats (Kemp et al., 2014, 2016). Chronic CGP42112A also reduced BP in obese hypertensive Zucker rats (Sabuhi et al., 2011; Ali et al., 2013) but not in 2-kidney-1-clip hypertensive mice (Cervenka et al., 2008), prehypertensive Zucker rats (Dhande et al., 2013) or high-salt–fed mice (Wang Y et al., 2020). C21 also reduced mesenteric artery stiffness and improved endothelial relaxation in mesenteric arteries together with reduced aortic collagen and fibronectin levels (Rehman et al., 2012). Similarly, Paulis and colleagues (2012) showed that C21 partly reduced aortic wall thickness, stiffening, and collagen accumulation that occurred as a result of L-NAME–induced hypertension and normalized vascular collagen content when combined with an ARB; all effects were BP and NO independent. In contrast, there were BP-dependent effects related to ex vivo vascular function since AT_2R-mediated contraction of mesenteric arteries from SHR was converted to AT_2R-mediated relaxation when BP was reduced by antihypertensive therapy to normotensive levels in SHR (You et al., 2005). Chronic AT_1R blockade also unmasked AT_2R-mediated vasorelaxation in previously unresponsive aortic vessels from SHR or SHR-SP (Cosentino et al., 2005; Savoia et al., 2005) or human gluteal resistance arteries (Savoia et al., 2007), which is consistent with increased vascular AT_2R expression after such treatments (Cosentino et al., 2005; You et al., 2005; Savoia et al., 2007). Moreover, an AT_2R-mediated component to the aortic antihypertrophic effects of ARB treatment was implicated since PD123319 reversed these vasoprotective effects (Tea et al., 2000; Jones et al., 2004, 2012; Brassard et al., 2005).

5. Aortic Aneurysm. A potential protective role of the AT_2R in aortic aneurysms (AAs) has been studied using experimental models of thoracic and abdominal aneurysms. Using a genetic mouse model of thoracic AA based on the fibrillin-1 mutation that is the most frequent cause of Marfan syndrome (Fbn1$^{C1039G/+}$ mice), it was shown that additional knockout of AT_2Rs accelerated aneurysm growth and rupture (Habashi et al., 2011). Indirect evidence for a protective role of the AT_2R was further based on the observation made in this and another study (Te Riet et al., 2016) that RAS inhibition by AT_1R blockade, but not by ACE or renin inhibition, attenuated thoracic AA, suggesting that the beneficial effect of AT_1R blockade was due to elevated Ang II levels stimulating unopposed AT_2Rs. This assumption was supported by a much weaker effect of AT_1R blockade in AT_2R-deficient than in wild-type mice (Habashi et al., 2011). Inhibition of TGF-β–induced ERK1/2 MAPK signaling was identified as underlying, protective AT_2R-coupled mechanism. The AT_1R antagonist losartan (i.e., potential indirect AT_2R stimulation) has been tested in various clinical trials in patients with Marfan syndrome for the deceleration of aneurysm progression, unfortunately with inconclusive results. However, a very recent long-term follow up analysis of the COMPARE trial found a significant reduction of death, need for surgery, and severe complications in patients treated with losartan in combination with a beta blocker (van Andel et al., 2020). Evidence for the relevance of AT_2Rs in the human situation is further supported by increased AT_2R expression in human AA: in human abdominal AA lesions, AT_2Rs were mainly localized in the inflammatory infiltrates and endothelium of *vasa vasorum* (Kaschina et al., 2009), whereas in human thoracic AA, AT_2Rs were found in protective $CD8^+/AT_2R^+$- and $CD4^+/AT_2R^+$-T-lymphocyte populations, which were associated with inhibition of growth, apoptosis, and MMP-2 expression (Wang et al., 2015; Wang C et al., 2017).

Three recent studies investigated direct pharmacological stimulation of the AT_2R with C21 or NP-6A4 in AA. In an elastase model of abdominal AA, treatment with C21 prevented aortic dilatation by mechanisms including antiproteolysis, reduced inflammation and fibrosis, and downregulation of apoptotic pathways (Lange et al., 2018). Antiproteolysis and anti-inflammation were also observed by Sharma et al. (2020) in response to AT_2R stimulation with NP-6A4 in abdominal AA induced by Ang II treatment in ApoE$^{-/-}$ mice. However, these effects at the molecular level only led to decreased aortic stiffness but not to an attenuation of aneurysm formation. In a model of Marfan syndrome in mice, treatment with a very high dose of C21

(0.5 and 5 mg/kg per day i.p.) for 6 months had no effect at all on thoracic AA development, which could have been caused by loss of selectivity of C21 for the AT_2R over the AT_1R at these high doses (Verbrugghe et al., 2018).

Due to the inconsistent outcomes, more studies are warranted to obtain more conclusive results about the efficiency of targeting the AT_2R for the treatment of aneurysm.

6. Preeclampsia. In pregnancy, an activated RAS plays a key role in maintaining fetoplacental circulation (Wilson et al., 1980). Interestingly, in healthy, pregnant women, the elevated Ang II plasma levels do not lead to an increase in BP despite an increase in extracellular volume (Gant et al., 1973). Preeclampsia, which is defined as onset of hypertension and proteinuria (>300 mg/l) after gestational week 20, is still one of the most common complications and the worldwide leading cause of death in pregnancy (Phipps et al., 2019). A highly indicative pathomechanism of preeclampsia is restoration or even exaggeration of the pressor response to Ang II (but not to other vasoconstrictors), which can already be detected before onset of symptoms and which after delivery remains for more than 1 year (Aalkjaer et al., 1985; Stanhewicz et al., 2017). There is evidence from studies in various species that an increase in vascular, vasodilatory AT_2Rs contributes to the protection of normally pregnant females from developing BP from increased Ang II levels, whereas in preeclampsia, angiotensin receptor expression changes in favor of AT_1 Rs causing an enhanced vasoconstrictive phenotype (Burrell and Lumbers, 1997; Stennett et al., 2009; Pulgar et al., 2011; Mishra et al., 2018; Assersen et al., 2020; Mishra and Kumar, 2021). Similar observations have also been made in human biopsies from normally pregnant and preeclamptic women (Judson et al., 2006; Hladunewich et al., 2011; Stanhewicz et al., 2017). The hypothesis of decreased AT_2R expression as pathogenetic factor in preeclampsia is further supported by the existence of an AT_2R polymorphism, which reduces the expression of AT_2Rs and is associated with an increased incidence in preeclampsia (Li et al., 2015). Mechanisms responsible for the imbalance in AT_1R/AT_2R ratio in preeclampsia are still unknown and warrant investigation, as does the suitability of AT_2R stimulation as a therapeutic approach in preeclampsia with the goal to compensate for reduced AT_2R expression.

D. Cutaneous Disease

Since the first description of the existence of a local RAS in mammalian (including human) skin ~20 years ago, multiple studies have investigated its relevance for dermatological diseases and found evidence for its involvement in wound healing, hypertrophic scars/keloids, scleroderma, dystrophic epidermolysis bullosa, Dupuytren's disease, squamous cell carcinoma, melanoma, and psoriasis. These findings have been reviewed in detail in several recent articles (Bernasconi and Nyström, 2018; Aleksiejczuk et al., 2019; Hedayatyanfard et al., 2020; Silva et al., 2020).

Regarding AT_2R in skin, current experimental evidence is strongest for a role in wound healing, Dupuytren's disease, and melanoma.

1. Wound Healing. With regard to wound healing, several studies unanimously reported dynamic changes in angiotensin receptor expression during the different phases of wound healing, specifically, a stronger increase in AT_1Rs than AT_2Rs during early, proliferative phases of wound closure, whereas in the later remodeling phase, AT_2R upregulation was prominent (Bernasconi and Nyström, 2018; Aleksiejczuk et al., 2019; Silva et al., 2020). Such a pattern makes sense since in the early phases of wound healing and reepithelization, a proproliferative action of AT_1Rs is needed for accelerating wound closure. Consequently, in AT_1R-KO mice and in rats treated with an ARB, wound closure was delayed (Takeda et al., 2004; Yahata et al., 2006; Faghih et al., 2015). In contrast, in AT_2R-KO reepithelization was accelerated. This supports an antiproliferative effect of AT_2Rs, which may balance the proproliferative AT_1R effects but will be overruled by AT_1R-mediated proliferation because of much higher abundance of AT_1Rs in this phase of wound healing (Faghih et al., 2015). During the remodeling phase of wound healing, AT_2R expression is stronger and its antifibrotic, TGF-β-lowering properties seem essential for the formation of resilient scar tissue as indicated by the fact that skin from AT_2R-KO ruptures from lower tension than skin from wild-type mice (Faghih et al., 2015).

2. Dupuytren's Disease. The antifibrotic features of the AT_2R further motivated researchers to test the effect of treatment with C21 in a xenograft model of Dupuytren's disease in which palmar cord specimens were implanted under the dorsal skin of nude mice (Chisholm et al., 2017). Systemic (intraperitoneal) administration of C21 led to a significant inhibition of myofibroblast proliferation. In dermal fibroblasts, C21 inhibited various components of the TGF-β signaling cascade [CTGF, fibroblast-specific protein-1, TGF-β1, Suppressor of mothers against decapentaplegic (SMAD) 3/4], thus clearly showing an antifibrotic, AT_2R-mediated effect in skin (Chisholm et al., 2017).

3. Melanoma. The third skin disease for which preclinical data strongly support a therapeutic effect of AT_2R stimulation is malignant melanoma. Treatment of mouse and human melanoma cells in vitro by an AT_2R agonist decelerated transendothelial and "normal" migration (Martínez-Meza et al., 2019). In vivo, AT_2R stimulation attenuated lung metastases from melanoma cells (Martínez-Meza et al., 2019).

This study and the underlying anticancer signaling mechanisms are reviewed in more detail in Section VI.H.

Pathomechanisms related to the RAS in skin and potential therapeutic interventions are a dynamic field, with many findings only made during very recent years and with many more insights to be expected.

E. Lung

1. Pulmonary Hypertension and Fibrosis. The preclinical studies on pulmonary fibrosis and hypertension are of particular interest since idiopathic pulmonary fibrosis was the first indication that was selected for the clinical development of AT_2R agonists.

A therapeutic effect of AT_2R agonists has been demonstrated in models of monocrotaline (MCT)-induced (Bruce et al., 2015), bleomycin-induced (Rathinasabapathy et al., 2018), and hyperoxia-induced pulmonary fibrosis (Wagenaar et al., 2013). Although the MCT model is regarded as the standard model for PH and the bleomycin model as the standard model for pulmonary fibrosis, PH and pulmonary fibrosis develop in both models.

Bruce et al. (2015) induced pulmonary fibrosis in 8-week-old Sprague-Dawley rats by a single subcutaneous injection of MCT. After fibrosis had fully developed over a period of 2 weeks, animals were treated with 0.03 mg/kg per day C21 i.p. for another 2 weeks. Treatment with the AT_2R agonist reversed pulmonary fibrosis and prevented right ventricular fibrosis. These beneficial effects were blocked by the AT_2R antagonists PD123319 and by the MAS antagonist A779 (Asp-Arg-Val-Tyr-Ile-His-D-Ala), the latter likely a result of dimerization and cross-inhibition (Leonhardt et al., 2017).

Rathinasabapathy et al. (2018) induced pulmonary fibrosis in 8-week-old Sprague-Dawley rats by a single intratracheal installation of bleomycin. Treatment with C21 (0.03 mg/kg per day i.p.) was started either immediately or 3 days after induction of fibrosis and continued up to day 14. The study revealed that treatment with an AT_2R agonist prevented the development of fibrosis when applied from day 0 or halted its development when applied from day 3.

Remarkably, in both studies, right systolic ventricular pressure, which is linked to pulmonary BP, was increased in diseased animals but could be lowered by AT_2R stimulation. This finding is important since PH is the most common cause of death in patients with pulmonary fibrosis. Induction of pulmonary fibrosis by MCT or bleomycin also involved increased pulmonary vascular remodeling as evidenced by increased vessel muscularization, and in both cases this remodeling was ablated by C21 treatment (Bruce et al., 2015; Rathinasabapathy et al., 2018). These studies also demonstrated that the antifibrotic actions of AT_2R activation involve decreased expression of the profibrotic cytokine TGF-β and anti-inflammatory actions through reduced expression of proinflammatory cytokines and chemokines (Bruce et al., 2015).

Waagenar et al. (2013) performed two studies in a model of neonatal chronic lung disease (CLD), which is a common complication in prematurely born newborns due to mechanical ventilation or prolonged oxygen supplementation resulting in pulmonary inflammation, alveolar rarefaction, fibrosis, PH, and right ventricular heart failure. For the animal model of CLD, neonatal rats were continuously exposed to 100% oxygen over the first 10 days after birth. Treatment with the AT_2R agonist LP2/3 reduced pulmonary inflammatory cell infiltration, alveolar septal thickness, and arterial remodeling. It further reduced right ventricular (RV) hypertrophy, which indirectly points to a reduction of PH; however. there were no direct measurements of RV pressure performed in this study. Interestingly, the same group subsequently performed a study with a very similar design and reported that the AT_2 antagonist PD123319 not only abolished the beneficial effects of LP2/3 reviewed above, but when applied alone, it basically had the same effects as the AT_2R agonist, which made the authors assume that PD123319 has characteristics of a partial AT_2R agonist (Wagenaar et al., 2014). A beneficial effect of PD123319 was also reported in a model of bleomycin-induced lung fibrosis (Waseda et al., 2008).

2. Acute Lung Injury. Acute lung injury (ALI) was induced in rats via an experimental model that involved pulmonary lavage followed by mechanical ventilation (Menk et al., 2018). These procedures led to impaired gas exchange, lung edema, and pulmonary inflammation. Application of C21 during induction of ALI led to decrease in pulmonary inflammation due a reduction in TNF-α and IL-6 expression, but there was no beneficial effect of C21 on either gas exchange or edema during the study period (Menk et al., 2018). Thus, the clinical utility of AT_2R agonism in ALI is not apparent at this time.

F. Central and Peripheral Nervous Systems

1. Stroke. There is now overwhelming evidence from preclinical studies using a variety of rodent models of middle cerebral artery occlusion (MCAO) that AT_2R activation exerts beneficial effects in ischemic stroke. This line of research began with speculation that the beneficial effects of intracerebroventricular pretreatment with an ARB before experimental ischemic stroke in rats were in part mediated by the unopposed central AT_2Rs (Dai et al., 1999), and this idea was substantiated by several studies. Iwai et al. (2004) not only demonstrated that AT_2R-KO exposed to permanent MCAO exhibit larger cerebral infarcts and increased behavioral deficits when compared with wild-type mice but also that the neuroprotective

effects elicited by the ARB valsartan were attenuated by AT_2R deficiency. Next, Li et al. (2005) demonstrated that neuroprotection elicited by the ARB irbesartan post-transient focal ischemia in rats was inhibited by the AT_2R antagonist PD123177, and likewise, Faure et al. (2008) later demonstrated that candesartan-induced neuroprotection in a thromboembolic MCAO model was abolished by the AT_2R antagonist PD123319. These findings suggested that the neuroprotective effects of ARBs in ischemic stroke are due to unopposed actions of Ang II at AT_2Rs. It is also pertinent that AT_2R expression was upregulated in peri-infarct cortical and hippocampal regions after ischemic stroke (Makino et al., 1996; Kagiyama et al., 2003; Li et al., 2005).

Subsequently, investigations have focused on using AT_2R agonists in ischemic stroke and studies performed by seven independent groups of investigators have revealed significant beneficial effects of these agents. Essentially, these studies revealed reductions in cerebral infarct size and improvement in neurologic deficits when applying either CGP42112A or C21 intracerebroventricular or peripherally in rats and mice before or after stroke induction via several different methods (permanent MCAO, temporary MCAO, focal cerebral ischemia, endothelin-1–induced vaso-occlusion) and in normal or hypertensive animals (McCarthy et al., 2009, 2012; Lee et al., 2012; Joseph et al., 2014; McCarthy et al., 2014a,b; Min et al., 2014; Alhusban et al., 2015; Ma and Yin, 2016; Mateos et al., 2016; Schwengel et al., 2016; Bennion et al., 2018a; Ahmed et al., 2019). Although the preceding studies were performed in adult male rats, it is also apparent that C21 is beneficial against ischemic stroke in aged animals (Bennion et al., 2017; Ahmed et al., 2018a, 2019) and, importantly, in female animals (Eldahshan et al., 2019; Jackson-Cowan et al., 2021). Only in one study, which used C21 in a thromboembolic MCAO ischemic stroke model, were the protective effects considered moderate (Ishrat et al., 2019). As a whole, these findings indicate that activating AT_2Rs exerts powerful beneficial effects against ischemic stroke, and these studies have largely fulfilled the recommendations of the Stroke Treatment Academic Industry Roundtable (STAIR) group for preclinical stroke research (Albers et al., 2011). In contrast to ischemic stroke, investigations of AT_2R actions in hemorrhagic stroke [intracranial hemorrhage (ICH)] are limited to one study, which demonstrated that long-term administration of C21 via oral gavage to SHR-SP delayed the appearance of intracranial damage and prolonged survival (Gelosa et al., 2009).

The underlying protective mechanisms involved in the beneficial effects of AT_2Rs in ischemic stroke are multimodal. These have been reviewed in detail previously (Bennion et al., 2018b) but essentially encompass neuroprotective, vascular, and restorative/regenerative actions. From a protective standpoint, AT_2R activation in ischemic stroke elicits anti-inflammatory, antichemotactic, antiapoptotic, and antioxidative/nitrative stress actions (Joseph et al., 2014; McCarthy et al., 2014b; Min et al., 2014; Alhusban et al., 2015; Ma and Yin, 2016; Schwengel et al., 2016; Fouda et al., 2017; Shan et al., 2018). The vascular actions of AT_2R activation include improvement of cerebral blood flow and also restoration of blood-brain barrier (BBB) integrity (Min et al., 2014), the latter being compromised in ischemic stroke (Kassner and Merali, 2015). The restorative/regenerative actions of AT_2R activation in ischemic stroke include increased production of brain-derived neurotrophic factor (BDNF), vascular endothelial growth factor (VEGF) (Alhusban et al., 2013, 2015; Mateos et al., 2016; Schwengel et al., 2016), and associated proneurotrophic, proneurogenesis, and proangiogenic actions. Given that the pathological mechanisms of ischemic stroke are multiple (Deb et al., 2010; Xing et al., 2012) and that the STAIR group recommend that therapeutics for ischemic stroke should have more than one mechanism of action (Albers et al., 2011), AT_2 R agonists that exert beneficial actions in ischemic stroke in a multimodal manner might present a viable therapeutic strategy for human stroke. Despite this and also that AT_2R agonists can be delivered to the brain and be effective via a clinically relevant nose to brain route (Bennion et al., 2018a), there has been no move to test their clinical efficacy in human ischemic stroke.

2. Brain Injury. Traumatic brain injury is a major health problem with unmet therapeutic need (GBD 2016 Traumatic Brain Injury and Spinal Cord Injury Collaborators, 2019), and a number of studies have indicated that novel treatment strategies may be found within the RAS, as previously reviewed (Janatpour and Symes, 2020; Mirzahosseini et al., 2021; Vadhan and Speth, 2021). Included within these are investigations that indicated that activation of AT_2R is beneficial in traumatic brain injury. In one study, intracerebroventricular delivery of CGP42112A into mice for 3 days after closed head injury produced improvements in functional recovery and cognitive performance versus controls, effects that were abolished by PD123319 (Umschweif et al., 2014a). Associated with these improvements, CGP42112A elicited activation of the neuroprotective kinase Akt and also the neurotrophins BDNF and nerve growth factor, suggesting that the beneficial actions of AT_2R activation involved both neuroprotection and neurogenesis (Umschweif et al., 2014a). The same group of investigators demonstrated that heat acclimation-induced neuroprotection in mice that had undergone closed head injury was partly mediated through AT_2R activation (Umschweif et al., 2014b). A more recent study

demonstrated that peripheral administration of C21 after unilateral cortical traumatic brain injury in mice elicited a significant reduction in neurologic deficits, an effect that was associated with decreased inflammation and apoptosis within the peri-contusional area of the brain (Ismael and Ishrat, 2021). Beneficial effects of AT_2 R agonism in brain injury are not restricted to traumatic brain injury, as C21 treatment lessens the neuronal apoptosis and synaptic loss induced by isoflurane anesthesia of neonatal rats (Yong et al., 2018). Collectively, these findings suggest that AT_2R activation can be beneficial in brain injury situations in adults and developing animals and that this area bears further investigation.

3. Spinal Cord Injury. The effects of C21 on neurologic performance and neuroregeneration have been examined after spinal cord compression injury in mice. Daily intraperitoneal (i.p.) injection of C21 for 4 weeks not only improved locomotor performance but also increased the number of corticospinal neuron fibers caudal to the lesion when compared with vehicle-treated mice (Namsolleck et al., 2013). This C21-treatment also increased the number of viable neurons and elicited increased expression of tropomyosin receptor kinase B (TrkB), the high-affinity receptor for BDNF, within and surrounding the lesion site (Namsolleck et al., 2013). The conclusion was that AT_2R activation exerted beneficial effects in spinal cord injury via neuroprotection and neuroregeneration.

4. Dementia. The most common causes of dementia, in which symptoms include cognitive decline and memory loss, are AD (2020 Alzheimer's disease facts and figures, 2020) and vascular dementia (Iadecola, 2013), the latter referred to as 'vascular cognitive impairment' in early stages of the disease (Wiesmann et al., 2013). As with stroke and traumatic brain injury, there is great unmet therapeutic need for AD and vascular dementia, and the RAS, including AT_2 Rs, may present opportunities for novel therapies based on preclinical studies (Mogi and Horiuchi, 2009; Wright and Harding, 2019; Ahmed and Ishrat, 2020). For example, the memory loss and reduction in cerebral blood flow induced by the antimuscarinic agent scopolamine in mice was ameliorated by the ARB candesartan, partly through AT_2R activation (Tota et al., 2012). Furthermore, beneficial actions of direct agonist-induced AT_2R activation have been demonstrated in several animal models of vascular dementia (Mogi et al., 2006; Iwanami et al., 2015; Ahmed et al., 2018a,b, 2019, 2022; Jackson et al., 2020; Eldahshan et al., 2021) and in AD (Jing et al., 2012; Ahmed et al., 2022). C21 has also been shown to act synergistically with the N-methyl-D-aspartate (NMDA) receptor inhibitor memantine (used clinically in severe AD cases) to prevent cognitive decline in type 2 diabetic mice (Iwanami et al., 2014). Despite these findings, other studies suggest that AT_2R activation may not be so beneficial in AD. One study demonstrated that Ang II activation of AT_2R alters amyloid precursor protein (APP) metabolism in senescent human microvascular endothelial cells and in doing so contributes to the development of cerebral amyloid angiopathy and thus to AD pathology (Sun et al., 2018). Furthermore, a recent extensive study employed the APP J20 mouse model of AD and indicated that although AT_2Rs were partly responsible for the cognitive, cerebrovascular, and antioxidant benefits of treatment with the ARB losartan, direct AT_2R activation with C21 provided few benefits in this regard (Royea et al., 2020). The authors concluded that targeting AT_2R alone was not an ideal intervention in AD (Royea et al., 2020). Considering the disparity of opinions as to whether AT_2R activation is beneficial in AD, further experimentation is warranted in this area.

5. Neuromyelitis Optica. Neuromyelitis optica spectrum disorder (NMOSD) is an autoimmune, inflammatory, and demyelinating CNS disease, which has in the past been regarded as a specific subtype of multiple sclerosis (MS) but is now defined as a separate entity with autoantibodies directed against the aquaporin-4 water channel (AQP4) being the main diagnostic criterion (Weinshenker and Wingerchuk, 2017). NMOSD primarily presents as inflammation of the optic nerve (optic neuritis) and spinal cord and leads to characteristic symptoms such as ocular pain and vision loss as well as impairment of motor, sensory, and autonomic functions primarily in the lower parts of the body. AT_2R stimulation was tested recently in an NMOSD model in which NMOSD-like pathological changes were induced in brains of C57BL/6 mice by intracerebral injection of anti-AQP4 antibodies (AQP4-IgG) derived from a patient with NMOSD together with complement (Khorooshi et al., 2020). Mice were treated either with the AT_2R-agonist C21 or vehicle by intracerebral coinjection with AQP4-IgG on day 1 and a second intrathecal injection on day 2. Analysis of brains on day 4 revealed protection from NMOSD-related astrocyte damage as shown by AQP4 and glial fibrillary protein staining. Since the treatment with C21 induced an increase in IL-10 mRNA expression and because the treatment effect of C21 was absent in IL-10 deficient mice, the authors concluded that IL-10 is a main mediator of the protective effect of AT_2R-stimulation in NMOSD (Khorooshi et al., 2020). In a follow up study, the same group reported an increase in BDNF mRNA expression coinciding with an AT_2R-mediated amelioration of NMOSD-like pathology. However, since the therapeutic effect of AT_2R-stimulation with C21 was preserved in mice with astrocyte-specific, conditional knockout of BDNF, a causal involvement of BDNF in the protective effect of C21 could not be proven. It is possible, however, that neuronal BDNF, which was not affected by the knockout, was responsible for the protective effect (Khorooshi et al., 2021).

6. Cerebral Malaria. Cerebral malaria is characterized by increased leakiness of the BBB and cerebral microhemorrhages after adhesion of *Plasmodium falciparum*-infected red blood cells (Pf-iRBCs) to cerebral endothelial cells (Storm and Craig, 2014). In a study using human brain microvascular endothelial cells, it was demonstrated that Pf-iRBC induces activation of β-catenin that in turn disrupts interendothelial cell junctions, providing a potential mechanism for the leaky BBB in cerebral malaria (Gallego-Delgado et al., 2016). In the same study, they demonstrated that the AT_2R agonist CGP42112A inhibited the Pf-iRBC–induced activation of β-catenin and disruption of interendothelial cell junctions and was protective in a mouse model of cerebral malaria, reducing the number of hemorrhages and increasing survival (Gallego-Delgado et al., 2016). Conversely, AT_2R-KO were more susceptible to cerebral malaria, with an earlier disease onset and 100% incidence compared with controls (Gallego-Delgado et al., 2016). In summary, AT_2R activators may be useful therapeutically in cerebral malaria.

7. Diseases Related to Dopamine Dysfunction. Degeneration of the nigrostriatal dopamine neurons that arise in the substantia nigra (SN) of the ventral mesencephalon and project to the caudate nucleus-putamen (CP) within the striatum is responsible for the motor deficits that are characteristic of Parkinson's Disease (PD) in humans (Beitz, 2014; Balestrino and Schapira, 2020). As discussed in Section III.E.6, AT_2 Rs have been located within rodent and human SN and CP (Barnes et al., 1991; Lenkei et al., 1996), and on nigrostriatal dopamine neurons (Grammatopoulos et al., 2007). Considering the well established beneficial roles of AT_2Rs in neurodegenerative diseases such as stroke and traumatic brain injury, it is possible that AT_2Rs may serve similar roles in PD. However, data on whether AT_2Rs play a beneficial role in PD are conflicting. AT_2R activation has been shown to protect ventral mesencephalic (including SN) dopamine neurons from death induced by ROS activation (Grammatopoulos et al., 2005) and to induce the differentiation of mesencephalic precursor cells into dopamine neurons, potentially providing a source of these neurons for transplantation in PD (Rodriguez-Pallares et al., 2004). To the contrary, it was demonstrated that direct infusion of C21 into the striatum decreased dopamine synthesis (Mertens et al., 2010), the opposite of what would be needed in PD. In summary, as yet there are no definitive studies which have determined the efficacy of AT_2R agonists to exert beneficial actions in animal models of PD.

On a different tack, it is known that striatal dopamine signaling is important for producing strong feeding responses (Mogenson et al., 1988), and there is much evidence for a role of dopamine in perpetuating the compulsive feeding pattern of binge eating disorder (Bello and Hajnal, 2010). Following on from the previous observation that C21 infusion in the striatum decreased dopamine synthesis (Mertens et al., 2010), it was demonstrated that intrastriatal C21 infusion in type 2 diabetic (KK-Ay) mice that had been fasted for 2 days not only reduced striatal dopamine levels but attenuated the rebound increase in food intake after fasting (Nakaoka et al., 2015). The conclusion was that AT_2 R activation may be a way of preventing binge eating disorder via lowering brain dopamine levels.

8. Neurogenic Hypertension. Based on results of studies using AT_2R agonists and antagonists (Brouwers et al., 2015; Dai et al., 2015, 2016) and on AT_2R gene transfer (Blanch et al., 2014), it is apparent that activation of AT_2Rs within CNS cardiovascular control centers exerts BP lowering and sympatho-inhibitory effects in several animal models of neurogenic hypertension (Fig. 10). In general, these effects are more powerful than those observed in normotensive animals (see Section V.B), and AT_2R activation or overexpression was also shown to improve the impaired baroreflex function that occurs in hypertension of neurogenic origin (Blanch et al., 2014; Ruchaya et al., 2016; Speretta et al., 2019). Interestingly, although AT_2R overexpression in the NTS of SHRs or rats made hypertensive by high fat diet resulted in improved baroreflex function, there was no associated lowering of BP (Ruchaya et al., 2016; Speretta et al., 2019). Nonetheless, it can be argued that brain AT_2Rs represent an endogenous depressor mechanism that, given the right conditions and ability to target select neuronal populations, might be taken advantage of to lower BP in hypertension. Indeed, a recent study has localized at least one set of neurons that are important in these antihypertensive effects by demonstrating that AT_2R-mediated inhibition of GABAergic "pressor" neurons in the NTS is critical to the AT_2R-mediated lowering of BP during deoxycorticosterone acetate (DOCA)-salt–induced hypertension (Mohammed et al., 2021).

9. Multiple Sclerosis/Experimental Autoimmune Encephalomyelitis. MS is a chronic inflammatory autoimmune disease of the CNS involving various immune cells such as autoreactive T cells, B cells, microglia, and macrophages, which cause demyelination, axonal degeneration, and subsequently functional impairment of neurons leading to disability in the patient (Dendrou et al., 2015).

The RAS via AT_1R promotes CNS inflammation in a variety of pathological situations including hypertension, stroke, brain injury, and also MS (Platten et al., 2009; Stegbauer et al., 2009; Lanz et al., 2010; Elsaafien et al., 2020). Expression of receptors of the protective RAS is increased in MS lesions but with delay compared with the increase in AT_1Rs, which may hint to a compensatory mechanism beginning to operate (Stone et al., 2020). AT_2R stimulation has indeed been shown to milden neurologic deficits and the inflammatory

response in experimental autoimmune encephalomyelitis (EAE), an animal model of MS (Valero-Esquitino et al., 2015). A 4-week treatment with C21, which was started 3 days before immunization with a fragment of myelin oligodendrocyte glycoprotein, significantly reduced the area of spinal cord demyelination and inflammatory infiltrates, mainly the total and $CD4^+$ T cell fractions. Inhibition of demyelination by AT_2R stimulation was confirmed in aggregating brain cell cultures, as was inhibition of microglia activity that was also observed in vivo (Valero-Esquitino et al., 2015).

10. Peripheral Neuronal Injury. As discussed in Section III.E.6, AT_2R mRNA levels were increased in the DRG and sciatic nerve after transection of the latter, leading the authors to suggest that AT_2R-mediated actions are involved in Schwann cell-mediated myelination and in neuroregenerative responses of DRGs (Gallinat et al., 1998).

A beneficial role of the AT_2R after injury of peripheral nerves was demonstrated in two studies from the same group of investigators. In a first study they demonstrated that Ang II, acting via AT_2Rs, promoted axonal elongation in postnatal retinal explants and dorsal root ganglia in vitro and axonal regeneration of retinal ganglion cells after optic nerve crush in vivo (Lucius et al., 1998). In a second study they demonstrated that activation of AT_2Rs elicited functional recovery from severe neuronal injury in vivo (Reinecke et al., 2003). Specifically, in a rat model of sciatic nerve crush, axonal regeneration and myelination were enhanced and the recovery of sensorimotor function accelerated, demonstrating for the first time a functional neuronal regenerative effect of AT_2R stimulation in vivo (Reinecke et al., 2003). This effect most probably involved nuclear translocation of NF-κB leading to enhanced remyelination (Reinecke et al., 2003).

11. Pain. The role and activity of AT_2Rs in pain is controversial, with some studies concluding that AT_2 Rs induce pain whereas others conclude the opposite, as reviewed in detail recently (Pulakat and Sumners, 2020). The idea that the AT_2R can influence pain began two decades ago with a study which demonstrated that stimulation of brain AT_2Rs can alleviate pain in a mouse model of acetic acid–induced abdominal constriction (Georgieva and Georgiev, 1999). Around the same time, another study demonstrated that not only did AT_2R-deficient mice exhibit increased sensitivity to pain induced by tail flick or pinch but that they expressed lower levels of β-endorphin in the arcuate nucleus of the brain compared with wild-type mice (Sakagawa et al., 2000). More recent studies have indicated that AT_2R stimulation reduces neuropathic pain. In one case, beneficial effects exerted by the ARB candesartan in resiniferatoxin-induced neuropathic pain were due to generation of Ang II and stimulation of the AT_2R (Bessaguet et al., 2017), and in another study from the same group, C21 was protective against vincristine-induced neuropathic pain (Bessaguet et al., 2018). In an entirely different scenario, the mycobacterial polyketide mycolactone activated neuronal AT_2Rs, leading to potassium-dependent hyperpolarization that induced analgesic effects (Marion et al., 2014; Song et al., 2017). This is, apparently, the underlying mechanism of how the severe Buruli ulcers induced by *Mycobacterium ulcerans* in humans are painless, a mechanism with translational potential (Babonneau et al., 2019; Reynaert et al., 2019).

In stark contrast to the above studies, a number of investigations have concluded that AT_2R antagonism relieves pain, particularly in animal models of neuropathic pain. The earliest example of this view came from a study that used a series of derivatives (EMA300, EMA400, and EMA401) of the AT_2R antagonist PD123319 (EMA200). As shown by radioligand binding assays, these molecules are highly selective for the AT_2R over the AT_1R, with affinities in the mid-nM range for AT_2R (Smith et al., 2013b). EMA200, EMA300, and EMA400 elicited analgesia against mechanical hypersensitivity induced by unilateral chronic constriction injury (CCI) of the sciatic nerve, a rodent model of neuropathic pain (Smith et al., 2013b). The caveat is that neither in this nor in subsequent studies were the EMA compounds demonstrated to be AT_2R antagonists based on functional assays—an important point as PD123319 (EMA200) has partial agonist activity at AT_2R in several other situations (see Section IV.C). Although EMA300-induced analgesia was abolished in AT_2R-KO subjected to CCI, this only indicates an AT_2R-dependent mechanism of action but is not proof for an antagonistic effect of EMA300 (Smith et al., 2013a). An analgesic effect of the EMA compounds was further shown in models of prostate cancer–induced bone pain (Muralidharan et al., 2014), of hind paw inflammatory pain (Chakrabarty et al., 2013), and of unevoked or Ang II-induced mechanical or cold-induced hypersensitivity in a spared nerve injury model of neuropathic pain (Shepherd et al., 2018a,b; Shepherd and Mohapatra, 2019).

Mechanisms described to mediate the analgesic effect of AT_2R antagonism included: 1) blockade of p38 MAPK and p44/p42 MAPK activation either directly (Smith et al., 2013a) or through a reduction of elevated Ang II levels in the lumbar DRGs to attenuate augmented Ang II/AT_2R signaling (Muralidharan et al., 2014); 2) attenuation of the capsaicin response (Anand et al., 2013); or 3) inhibition of CD3+ T cell infiltration and increased nerve growth factor expression (Khan et al., 2017). The latest reported mechanism underlying the "pain-inducing" action of AT_2R was not a direct

effect on sensory neurons, as the authors failed to observe AT_2R on these cells (Shepherd et al., 2018a,b). Rather, their data indicate that AT_2R located on macrophages are responsible for the production of ROS/reactive nitrogen species which transactivate transient receptor potential subfamily A (TRPA1) channels on mouse and human DRG sensory neurons to elicit pain (Shepherd et al., 2018a,b).

The above-discussed preclinical studies using the EMA series of AT_2R antagonists precipitated a drug development program for EMA401 (the *S*-enantiomer of EMA400 chosen as lead for its high AT_2R selectivity and good oral bioavailability), with neuropathic pain as main indication. However, as reviewed in detail in Section VII, after an initial, successful phase II trial, a second series of trials was terminated prematurely due to preclinical toxicity of the compound (ClinicalTrials.gov Identifier: NCT03297294, NCT03094195) and without significant results regarding treatment effect (Rice et al., 2021).

Clearly, there is a huge discrepancy between the preclinical findings that indicate that AT_2Rs cause pain versus those that support a pain-reducing effect. This is an important issue with relevance regarding the rationale for developing further AT_2R antagonists for the treatment of pain and also regarding pain as potential adverse effect of AT_2R agonists, which may hamper their further development for idiopathic pulmonary fibrosis and COVID-19 (Section VII). However, so far in none of the clinical trials with an AT_2R agonist has pain been reported as an adverse effect. Several factors may be considered in attempting to resolve the discrepancy. First and foremost, none of the EMA compounds, particularly EMA401, have been tested in a functional AT_2R assay to assess whether they are pure antagonists or also possess agonistic activity. Given that C21, an established AT_2R agonist, is protective in neuropathic pain (Bessaguet et al., 2018), this is a critical point. A second factor that should be considered is that certain of the studies that concluded that AT_2R antagonists are beneficial in neuropathic pain used inflammatory or acute pain models that do not mimic chronic neuropathic pain (Pulakat and Sumners, 2020). Addressing these points would help to uncover whether AT_2R induces or alleviates pain or can exert both actions.

G. Metabolism

Metabolism is a net body function that produces energy and is coordinated by various organs such as the pancreas, liver, muscles, and adipose tissue. Insulin, an endocrine hormone, plays a central role in regulating metabolic activities, and an insufficiency or reduced insulin function leads to metabolic disorders such as diabetes, obesity, and fatty liver. Numerous studies have implicated involvement of AT_2Rs in various metabolic functions as summarized below (Fig. 12).

1. Glucose Metabolism.

a. Islet differentiation, function, and protection. The expression of AT_2Rs has been reported in various cell types of the pancreas, including insulin producing β-islets (for details see Section III.E.7). There are studies showing that AT_2Rs are involved in the differentiation of pancreatic progenitor cells (PCCs) into insulin-producing cells, thereby promoting insulin release and glycemic control (also under diabetic condition) (Leung et al., 2012, 2014). Specifically, Ang II via AT_2R activation induced differentiation of human fetal PCCs into islet-like cell clusters (ICCs), which are insulin producing β-cells, involving upregulation of the transcription factors neurogenin-3 (Ngn3) and pancreatic and duodenal homeobox 1 (Pdx1) (Leung et al., 2012). The in vivo relevance of these findings was demonstrated by transplantation of differentiated ICCs into mice with STZ-induced diabetes, which resulted in amelioration of hyperglycemia, whereas transplantation of AT_2R-depleted ICCs had no effect (Leung et al., 2012). These findings were confirmed by a follow up study in which treatment of pregnant mice with PD123319 led to a reduced number of β-cells in neonates and an impairment of their insulin secretory function (Leung et al., 2014). Prenatal exposure to dexamethasone is known as well to impair β-cell function in offspring, which interestingly could be restored by AT_2R overexpression in an in vitro model (Kou et al., 2020). In line with this, STZ-diabetic, C21-treated rat neonates had lower blood glucose, better insulin secretory function, and higher islet-cell mass than nontreated controls (Wang L et al., 2017), which could be due to AT_2R-mediated improved β-cell function or protection of islets from STZ toxicity as shown in adult rats (Shao et al., 2014). Collectively, these studies support a role for the AT_2R in islet cell differentiation, protection, and function.

b. Insulin sensitivity. In a broad understanding, insulin sensitivity is a measure of insulin function and signaling, which is negatively impacted by various factors such as obesity, increased adipocyte size, dyslipidemia and inflammation (Freeman and Pennings, 2021). Any improvement in these causative factors may improve insulin sensitivity or reduce insulin resistance, which is a hallmark of type 2 diabetes. AT_2R activation has been associated with improved insulin sensitivity in KK-Ay type 2 diabetic mice (Ohshima et al., 2012), in rats fed a high-fat/high-fructose diet (Shum et al., 2013), in healthy and STZ-diabetic rats (Shao et al., 2013, 2014; Begorre et al., 2017), in neonatal STZ-diabetic rats (Wang L et al., 2017), in mice with high-fat diet (HFD)-induced obesity/adiposity (Nag et al., 2015, 2019), in healthy, normal C57BL/6 mice (Quiroga et al., 2018), and in female diabetic db/db mice (Dominici et al., 2020). The beforementioned studies, which were based on AT_2R

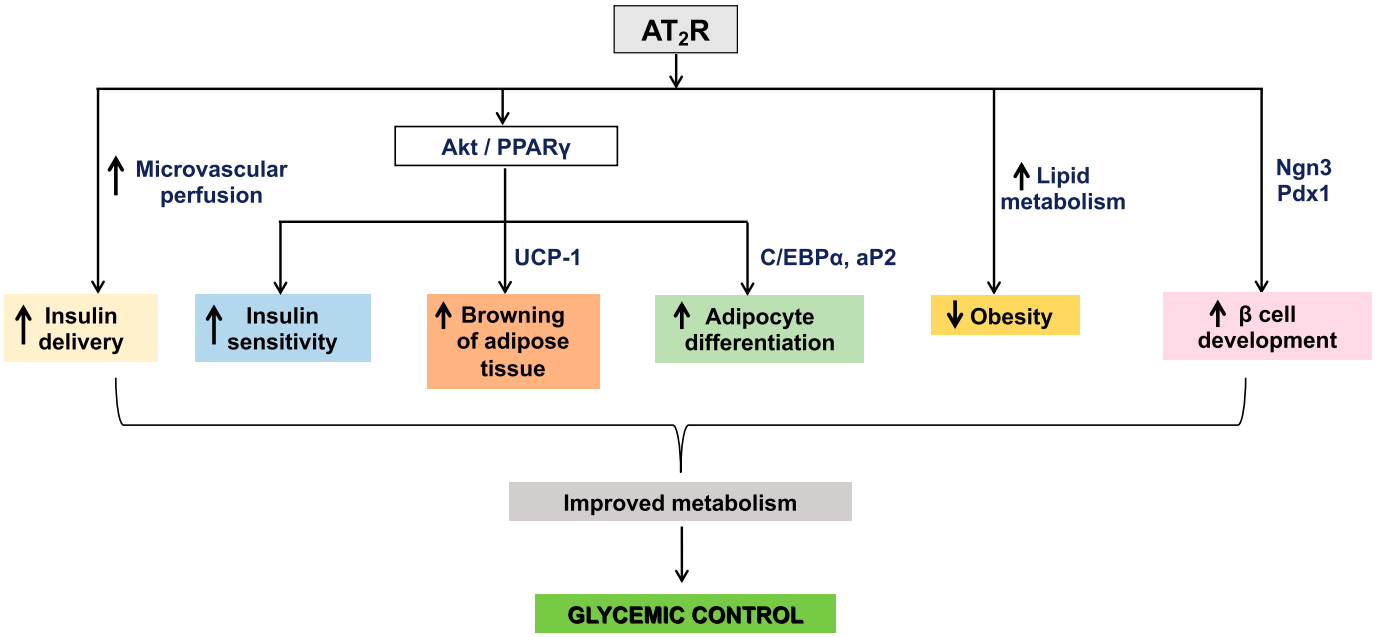

Fig. 12. Metabolic effects of AT_2R activation. AT_2R-stimulation promotes and improves various metabolic processes, including adipocyte differentiation and β-cell development that lead to better glycemic control in diabetic and obesity conditions. aP2, adapter protein 2; C/EBPα, CCAAT-enhancer–binding protein α; Ngn3, neurogenin-3; Pdx1, pancreatic and duodenal homeobox 1.

stimulation, are supported by a study in which chronic blockade of the AT_2R with PD123319 led to a worsening of determinants of insulin sensitivity (Muñoz et al., 2017). Mechanisms that were identified to mediate the AT_2R-induced improvement in insulin sensitivity included increased PPARγ expression (Ohshima et al., 2012; Shum et al., 2013) as well as a reduction in obesity/adiposity, dyslipidemia, and adipose tissue inflammation, which may have indirectly influenced insulin sensitivity. However, there is also evidence for a direct, positive interaction of the AT_2R with insulin signaling through phosphorylation of the insulin receptor, Akt, and forkhead box O1 (FOXO1), as shown in the liver of C21-treated female db/db mice (Dominici et al., 2020). This is supported by reduced insulin-induced phosphorylation of the insulin receptor or of Akt in adipose tissue or skeletal muscle of PD123319-treated or AT_2R-deficient mice (Chai et al., 2011; Muñoz et al., 2017; Quiroga et al., 2019).

Although the above-reviewed studies uniformly indicated a beneficial role of AT_2Rs in insulin signaling and sensitivity, studies in AT_2R-KO provided inconclusive results but pointed to sex differences. Two groups reported a deterioration of glucose metabolism in female but not male AT_2R-KO. In one of these studies, AT_2R deficiency in female mice led to increased body weight gain and impaired glucose tolerance coinciding with reduced estrogen levels (Samuel et al., 2013). In the other study, female AT_2R-KO presented with decreased insulin sensitivity potentially caused by reduced insulin resistance and Akt phosphorylation in adipose tissue (Quiroga et al., 2019). However, in both studies, knockdown of the AT_2R in male mice either had no effect or improved glucose metabolism and weight gain, thus supporting a neutral or detrimental effect of the AT_2R in physiological glucose homeostasis (Samuel et al., 2013; Quiroga et al., 2019). An unfavorable role of the AT_2R on insulin sensitivity was also observed in male AT_2R-KO on a high-fat diet (Yvan-Charvet et al., 2005).

Overall, targeting the AT_2R with pharmacological tools clearly supports a favorable role in glucose metabolism and insulin function, particularly in adipose tissue. However, data from AT_2R-KO are controversial and support a beneficial role only in female animals. It may be interesting to explore the molecular basis for the different metabolic outcome in pharmacological versus knockout studies, which is apparent in other models such as cardiac hypertrophy as well (Steckelings et al., 2010).

c. Muscle perfusion and glucose uptake. Microvascular perfusion of muscles is one of the determinants of insulin delivery and function (Clark, 2008). Through their vasodilatory effect (see Section V.C), AT_2Rs likely increase blood volume delivered to muscles. Indeed, C21 infusion caused an increase in microvascular blood flow in muscle associated with an increased insulin delivery to skeletal and cardiac muscle (Yan et al., 2018). In line with this finding, acute systemic blockade of AT_2Rs with PD123319 caused an inhibition of muscular glucose uptake in rats infused with Ang II or in overnight-fasted rats infused with insulin (Chai et al., 2010, 2011). However, in healthy AT_2R-KO, muscular glucose

uptake was unchanged compared with wild-type mice (Shiuchi et al., 2004).

2. Fat Metabolism.

a. Obesity and adipocyte differentiation. Obesity, a main risk factor for cardiovascular disease and diabetes, is characterized by excess white adipose tissue (WAT). Mechanisms that drive an enhancement of WAT mass are an increase in adipocyte size as well as adipogenesis, which is the differentiation of preadipocytes to mature, white adipocytes capable of fat storage (Ghaben and Scherer, 2019). Obesity is associated with adipose tissue inflammation and abnormal secretion of adipokines. Several studies have been performed in obesity models to investigate the role of the AT_2R on body weight gain and on the various pathological features of obesity described above.

Studies reporting AT_2R-mediated effects on body weight are controversial. Although AT_2R stimulation in models of type 2 diabetic KK-Ay mice (Ohshima et al., 2012), obese Zucker rats on a high-salt diet (Ali et al., 2015), and mice on a HFD reported an attenuation of body weight gain, several other studies using pharmacological AT_2R stimulation in models of obese Zucker rats (Dhande et al., 2013; Patel et al., 2016), mice fed a HFD (González-Blázquez et al., 2021b), or diabetic db/db mice (Dominici et al., 2020) saw no effect. Negative results may have been due to an insufficiently long treatment period since in female mice fed a HFD, an attenuation of weight gain by C21 was apparent after 12 weeks but not after 2 weeks (Nag et al., 2015). Interestingly, despite no difference in total body weight, parametrial WAT weight was already significantly reduced in C21-treated animals in this study after 2 weeks. A comparable result of no difference in body weight but significant reduction in epididymal WAT was obtained in male mice on a 2-week HFD (Nag et al., 2019).

Both studies, which reported a decrease in WAT weight, also found an AT_2R-mediated reduction in adipocyte size; an improvement of markers of lipid metabolism; a reduction in plasma insulin, fatty acids, and inflammatory mediators; and improved glucose tolerance (Nag et al., 2015, 2019). Favorable effects on adipose tissue homeostasis in response to AT2R stimulation, for example attenuated obesity-related inflammation (Sabuhi et al., 2011; Ohshima et al., 2012; Begorre et al., 2017) or improved adipokine generation (e.g., increased adiponectin levels) (Ohshima et al., 2012; Than et al., 2017; Quiroga et al., 2018; Dominici et al., 2020) were reported by several other studies regardless of any effect on body weight.

Adipocyte differentiation and smaller size are important for normal lipid storage and for protection against obesity and insulin resistance (McLaughlin et al., 2007). In addition to the studies mentioned above, reduced adipocyte size in response to C21 was also reported in type 2 diabetic KK-Ay mice (Ohshima et al., 2012), C57BL/6 mice (Quiroga et al., 2018), and rats on high-fat/high-fructose diet (Shum et al., 2013). Reduced adipocyte size was associated with increased PPARγ expression and DNA binding activity and with elevated adipocyte differentiating markers such as CCAAT-enhancer–binding protein α (C/EBPα) and adipocyte protein 2 (aP2) (Ohshima et al., 2012; Shum et al., 2013).

Direct evidence for a role of the AT_2R in promoting adipocyte differentiation was provided by in vitro experiments in preadipocytes in which C21 increased expression of PPARγ and aP2 and preserved a smaller cell and droplet size (Shum et al., 2013). These effects were reversed by short hairpin RNA (shRNA) knockdown of the AT_2R. Studies in human adipose mesenchymal cells also suggested that AT_2R activation promotes adipogenic differentiation of mesenchymal cells (Sysoeva et al., 2017).

Contrary to these pharmacological studies, in AT_2 R-KO studies the AT_2R inhibited adipocyte differentiation (Matsushita et al., 2016; Sysoeva et al., 2017).

b. White and brown adipose tissue. WAT is responsible for energy storage in the form of triglycerides, whereas brown adipose tissue (BAT) is a source of thermogenesis, energy dissipation, and metabolism. Brown-like adipocytes (beige adipocytes) can also be found in WAT and are identifiable by expression of thermogenic genes such as uncoupling protein-1 (UCP-1). There is evidence that the AT_2R shifts the WAT/BAT ratio in favor of BAT. For example, in mouse and human primary white adipocytes, AT_2R activation increased expression of UCP-1 and of Cbp/P300-interacting transactivator 1 (CITED1), a specific beige adipocyte marker, together with morphologic browning of cells (Than et al., 2017). An AT_2R-mediated increase in UCP-1 was further reported for C57BL/6 mice in vivo (Quiroga et al., 2018), as was a prevention of an HFD-related decrease in UCP-1 expression in BAT in another study in mice (Nag et al., 2019). The adipocyte browning effect involved increased PPARγ expression and ERK1/2 MAPK, Akt, and AMP-activated protein kinase (AMPK) signaling (Than et al., 2017; Quiroga et al., 2018). A shift toward a BAT-enriched adipose tissue composition allowing higher energy dissipation was supported by an increase in body temperature in C21-treated mice (Than et al., 2017; Nag et al., 2019).

c. Lipotoxicity and polycystic ovary syndrome. Nonesterified fatty acids (NEFA), if not stored in adipose tissue, produce lipotoxicity and are believed to be a cause of hyperandrogenemia and insulin resistance, which are involved in the pathogenesis of polycystic ovary syndrome, a condition affecting 6%–8% of women of childbearing age (Connolly et al., 2018). In a study in female JCR:LA-cp/cp rats, an

obese model of polycystic ovary syndrome exhibiting hyperandrogenism, insulin resistance, and polycystic ovaries, C21 treatment of 7 days significantly lowered elevated testosterone levels and ovarian NEFA uptake and C21 treatment also improved blood insulin and NEFA levels but these latter effects were not statistically significant, perhaps due to the short duration of treatment (Leblanc et al., 2014). This assumption is supported by a significant reduction in NEFA levels in mice fed an HFD after 6 weeks of treatment with C21 (González-Blázquez et al., 2021b). In line with the C21-induced reduction of NEFA-uptake, a greater NEFA-uptake by nonadipose tissues was reported in AT_2R-KO compared with wild-type mice (Noll et al., 2015).

d. AT_2R agonist effect on plasma lipoprotein levels in humans. Since no AT_2R-targeting drugs have been approved for clinical use as yet, data about metabolic effects of AT_2Rs in humans are scarce. An exception are data from an extension of the phase I, randomized, double-blind, placebo-controlled clinical trial with C21, in which young overweight (BMI 30–35 kg/m2; waist/hip >0.90) but otherwise healthy male volunteers were treated for 1 week with C21 (100mg once daily) or placebo. Metabolic markers were measured by quantitative serum nuclear magnetic resonance metabolomics. It was found that in the C21-treated group, LDL levels were consistently reduced and that high-density lipoprotein (HDL) levels increased between day 1 (before start of treatment) and day 8 (after last dose) of the trial (Steckelings et al., 2018). These changes did not occur in the placebo-treated subjects.

H. Cancer

Due to its antiproliferative, differentiation-promoting, and proapoptotic effects, early investigations hypothesized that AT_2Rs may play a role in the pathogenesis of cancer and represent a potential therapeutic target. This assumption is now supported by observations which demonstrate that AT_2R activation modulates a number of anticarcinogenic signaling pathways. These include stimulation of the intrinsic and extrinsic proapoptotic signaling pathways, activation of PP2A (Eichhorn et al., 2009; Perrotti and Neviani, 2013) and protein tyrosine phosphatase 1B (PTP1B) (Martínez-Meza et al., 2019), interference with mitotic spindle formation, and inhibition of histone deacetylase 1 (HDAC1) with subsequent activation of p53 (Peluso et al., 2022).

Moreover, ATIP, whose association with the AT_2R leads to inhibition of proliferation (see Section III.C.1.b) (Nouet et al., 2004; Wruck et al., 2005), was simultaneously and independently identified as a tumor suppressor, pointing again to an anticancer effect of AT2Rs (Seibold et al., 2003). ATIP is coded for by the microtubule-associated tumor suppressor gene Mtus1 (Haykal et al., 2021), and as reviewed elsewhere (Rodrigues-Ferreira and Nahmias, 2010; Bozgeyik et al., 2017), numerous studies have demonstrated that reduced levels of Mtus1 are associated with the development, progression, metastasis, or a poorer prognosis of a variety of cancers in humans, animal xenograft models, and in vitro. Included among the Mtus1-dependent cancers are bladder, breast, colorectal, gallbladder, head-and-neck, gastric, ovarian, lung, oral, and renal cancers as well as uveal melanoma (Haykal et al., 2021).

A number of splice variants of ATIP have been identified (ATIP1, ATIP3, ATIP4), of which ATIP3 is best characterized for its tumor suppressor properties. Whether ATIP3 binds to the AT_2R is currently not known, although it possesses the C-terminal amino-acid sequence necessary for AT_2R binding (Nouet et al., 2004). ATIP1/AT_2R binding is well established but seems to induce different signaling mechanisms, though still antiproliferative, from ATIP3/Mtus1 (Haykal et al., 2021). ATIP3 is probably most thoroughly studied in breast cancer, in which its expression levels were frequently reported to be decreased, a change associated with a higher aggressiveness of the tumor (Rodrigues-Ferreira et al., 2009, 2020). In preclinical studies, it was shown that silencing of ATIP3 promoted tumor cell proliferation, whereas delivery of Mtus1-transfected breast cancer cells into xenograft models led to inhibition of tumor growth (Rodrigues-Ferreira et al., 2009) and to a reduction of metastatic colonization (Molina et al., 2013). Both of the aforementioned studies identified an interaction between ATIP3 and microtubules leading to prolongation of metaphase as antiproliferative mechanism. The interference of ATIP3 with microtubule depolymerization and spindle dynamics seems to involve an interaction with the kinesin family member 2A (KIF2A) and its regulator, Dda3 (Nehlig et al., 2021).

In renal cell carcinoma, ATIP3-dependent microtubule stabilization involved phosphorylation of kinesin family member 2C (KIF2C), which is another microtubule depolymerizing kinesin (Lv et al., 2020). In salivary adenoid cystic carcinoma, downregulation of Mtus-1/ATIP3 was associated with enhanced migration and invasion of cancer cells and a poor outcome (Zhao T et al., 2015), and in squamous carcinoma of the tongue, MTUS/ATIP3 suppresses the proliferation and migration of invading cancer cells (Zhao et al., 2016). In addition to activation of ATIP, some other anticancer mechanisms have also been reported to be activated by AT_2Rs, all of them eventually resulting in inhibition of proliferation or induction of cell apoptosis. For example, a study in various human bladder cancer cell lines reported antiproliferation (shown by WST-1 assay) and apoptosis (shown by changes in

cell morphology and TUNEL staining) in response to transfection with an AT_2R-expressing adenovirus (Ad-GAT2R-eGFP) when compared with transfection with a non–AT_2R-expressing virus (Pei et al., 2017). To look more closely into proapoptotic mechanisms, the authors performed an Apoptosis Profile PCR Array and found the expression level of 11 of the 84 genes covered by the array to be changed in response to AT_2R stimulation. Analysis of the array and additional western blot experiments identified a proapoptotic signaling pathway involving activation of p38 MAPK, caspase-8, and caspase-3 and downregulation of ERK1/2 MAPK. Applying the apoptosis array to human prostate cancer cells, the same authors identified Gadd45a (growth arrest and DNA damage–inducible 45 alpha gene; mediator of cell cycle inhibition), TRAIL-R2 (receptor for the apoptosis-inducing ligand TRAIL), and HRK (proapoptotic BH3-only Bcl-2 family member, harakiri) to be crucially involved in AT_2R-mediated apoptosis (Pei et al., 2014). In contrast to activation of proapoptotic AT_2R signaling in bladder cancer cells, the proapoptotic pathway identified in prostate cancer cells was independent from of p38 MAPK, p44/42 MAPK, and p53.

A proapoptotic effect involving caspase 3 activation was also reported for human lung adenocarcinoma cell line A549 and bronchioloalveolar carcinoma line H358 after transfection with AT_2Rs using adenoviral, FuGENE, and nanoparticle vectors (Pickel et al., 2010). Lewis lung carcinoma is another type of pulmonary malignancy, which reacted to AT_2R overexpression with a reduction in growth and an increase in cell apoptosis. This was shown in studies that tested novel techniques of nonviral transfection such as intratracheal or intravenous application of synthetic cell-penetrating peptide (CPP) (polylysine, K9 peptide)/AT_2R plasmid DNA complexes or nanoparticle vectors (Kawabata et al., 2012; Alhakamy et al., 2016).

Involvement of p53 in the apoptotic effect of the AT_2R is controversial. Data for and against its involvement exist even for the same type of cancer (i.e., prostate cancer) (Li et al., 2009; Pei et al., 2014). A role for p53 in AT_2R-mediated apoptosis was also described for normal endothelial cells. In this study, p53 was activated by AT_2R-mediated inhibition of HDAC1 (another anticancer mechanism) and subsequent prevention of deacetylation of p53 (Peluso et al., 2022).

Although the above-reviewed studies in prostate and bladder cancer were performed using adenoviral overexpression of the AT_2R, a more recent study applied the AT_2R agonist C21 for treatment of human LNCaP prostate cancer cells and TRAP (transgenic rats for adenocarcinoma in prostate) rats (Ito et al., 2018). The authors confirmed the antiproliferative and proapoptotic effect of AT_2R stimulation involving caspase 3/7 activation in vitro and in vivo. In addition, they identified a novel anticancer mechanism of AT_2R stimulation in prostate cancer (i.e., a decrease in androgen receptor expression) (Ito et al., 2018). The group of Culman published two articles on the effect of AT_2R stimulation in serum-deprived, quiescent human, uterine leiomyosarcoma SK-UT-1 cells and suggested a mitochondrial, proapoptotic mechanism in these cells, which presented with a high density of AT_2Rs in mitochondria (Zhao et al., 2016; Lützen et al., 2017).

Treatment of leiomyosarcoma cells with the AT_2R agonist C21-induced rapid cell death within 24 hours and activated the intrinsic, mitochondria-dependent apoptotic pathway (Zhao Y et al., 2015). In contrast, leiomyosarcoma cells cultured under addition of 10% FBS expressed negligible amounts of AT_2Rs and did not undergo apoptosis. In the second study, AT_2Rs on SK-UT-1 cells were stimulated with Ang II under concomitant AT_1R blockade, which at early time points ($\leq$6 hours) inhibited proliferation and promoted differentiation as shown by an increase in the smooth muscle cell differentiation markers and tumor suppressors calponin and SM22α, whereas sustained AT_2 R stimulation ($\geq$36 hours) activated the intrinsic apoptotic pathway by a PPARγ-dependent mechanism (Lützen et al., 2017).

Inhibition of angiogenesis is another anticancer mechanism that has been ascribed to the AT_2R with an inhibition of VEGF expression as underlying mechanism as described (e.g., in bladder cancer) (Pei et al., 2017). In addition to AT_2R overexpression or application of an AT_2R agonist, another approach used in AT_2R-related cancer studies are AT_2 R-deficient mice. For example, Doi et al. (2010) reported that xenografts from mouse pancreatic ductal carcinoma cells grew faster in AT_2R-KO than in wild-type mice. The authors confirmed this indirect evidence for an anticancer effect of AT_2R stimulation in a model of pancreatic ductal carcinoma in a follow-up study using the AT_2R agonist $[Y]^6$-Ang II and AT_2R adenovirus transfection (Ishiguro et al., 2015).

In general, it is interesting that AT_2R stimulation seems to act in a proapoptotic manner mainly in tumor cells and much less so in the respective benign cells (Ishiguro et al., 2015; Zhao Y et al., 2015).

I. Eye

1. Retinal Inflammation. Retinal inflammatory disease is a dysfunction of the retina that is associated with increased production of proinflammatory cytokines and may result in vision loss (De Vos et al., 1992). As discussed in Section III.E.11, AT_2Rs are present in the retina in rodents and humans. Given the potent anti-inflammatory effects of AT_2R activation

(Patel et al., 2020), two studies have investigated whether activation of retinal AT_2Rs can produce beneficial effects in experimental models of retinal inflammation. In one study it was demonstrated that the inhibitory effects of the ARB telmisartan on the reduction in synaptophysin in retinal inflammation in mice were not only due to AT_1R-blockade but also due to upregulation of AT_2R function (Kurihara et al., 2006). In a more recent study, Verma et al., (2019) demonstrated, using cultured human retinal pigment epithelial (RPE) cells, that the AT_2R agonist C21 exerted potent inhibitory effects on the increases in proinflammatory cytokines and NF-κB produced by treatment of cells with Ang II, H_2O_2, or lipopolysaccharide (LPS). In the same study, C21 was shown to inhibit Ang II- and H_2O_2-induced ROS generation (Verma et al., 2019). Collectively, these preclinical studies suggest the potential utility of AT_2R agonists in retinal inflammatory disease.

2. Retinopathies. Retinopathies, whether induced by diabetes or associated with prematurity, share a common pathophysiological feature of growth factor–induced angiogenesis that can lead to at least retinal detachment and potentially to blindness (Hellström et al., 2013; Wang and Lo, 2018). Investigations on the role of the RAS in retinopathies have primarily focused on deleterious actions of Ang II via its AT_1R, and the few studies on AT_2R have thus far yielded inconclusive results (Fletcher et al., 2010). Oxygen-induced retinopathy (OIR) is often used as an experimental model to mimic retinopathies, and OIR produces profound VEGF expression and angiogenesis (Smith et al., 1994; Sarlos et al., 2003). In one study in mice, OIR-induced retinal angiogenesis was unaffected by the AT_2R antagonist PD123319 (Lonchampt et al., 2001), whereas in a similar study in rats PD123319 attenuated angiogenesis in OIR (Sarlos et al., 2003). The latter study also reported that the OIR-induced increases in retinal VEGF and VEGF receptor 2 (VEGFR-2) were reduced by AT_2R antagonism (Sarlos et al., 2003), consistent with a study that demonstrated that increased retinal VEGF gene and protein expression in diabetic rats was abolished by PD123319 (Zhang et al., 2004). Thus, in the case of retinopathies, the available data suggest that AT_2R may potentially exert deleterious effects by increasing VEGF expression and angiogenesis, unlike the beneficial effects gained by stimulating these processes in the brain after ischemic stroke (Alhusban et al., 2015; Mateos et al., 2016). However, it is difficult to conclude either way on the benefits or detriments of AT_2 Rs in retinopathies given the paucity of studies and the lack of any involving use of an AT_2R agonist. It also has to be taken into account that the proangiogenic effect was observed in a model in which retinopathy was driven by ischemia (and not inflammation), which may have caused an ischemia-induced response unrelated to the pathomechanism of retinopathy. Further studies are warranted, especially as inflammation has an essential role in the pathogenesis of retinopathy (Wang and Lo, 2018) and AT_2R agonists are powerful anti-inflammatory agents in general (Rompe et al., 2010b; Patel et al., 2020), including in the eye (Kurihara et al., 2006; Verma et al., 2019).

Key Points related to Section VI on the AT_2R in disease are:

- A large majority of experimental evidence indicates that stimulation of AT_2Rs exerts protective effects in a wide variety of disease states, in diverse tissues and organs. As depicted in the Visual Abstract that accompanies this article, there are established protective effects in cardiometabolic, vascular, lung, renal, and neural diseases and cancer. There are also other disease states where protective effects of AT_2R activation are likely, although more studies are needed to confirm these actions.
- The facts that AT_2Rs exert these beneficial actions and that AT_2R expression is often increased under disease conditions illustrate the point that these receptors probably represent an endogenous protective system that attempts to alleviate disease progress. As discussed in the next section (Section VII), this system has been taken advantage of with the use of a selective AT_2R agonist to exert protective effects in human disease.
- The actions of AT_2R in certain disease states are not without controversy, however. Both beneficial and detrimental actions of AT_2R have been observed in pain and in cardiac hypertrophy, whereas in AD AT_2R has been shown as beneficial or without effect. In the case of pain, which is the most controversial as experimental studies had progressed to clinical trials of AT_2R antagonists (Section VII), there needs to be a resolution as to whether the "AT_2R antagonists" used are pure inhibitors or are partial agonists.

VII. AT_2-Receptor Agonists and Antagonists in Drug Development Programs

Almost 20 years of preclinical research targeting the AT_2R in multiple disease models has produced strong evidence for the therapeutic efficacy of AT_2R ligands in many conditions (reviewed in the preceding sections). Consequently, a number of projects to develop AT_2R-targeting molecules for clinical use have

been initiated. AT_2R-targeting molecules that were successfully tested in phase I clinical trials include the agonists C21 (EudraCT No. 2015-005718-32; Vicore Pharma, Sweden) (Steckelings et al., 2017a); MOR-107 (also termed LP-2/3; Morphosys, Germany) (ClinicalTrials.gov Identifier: NCT03067363); and the antagonist EMA401 (EudraCT No. 2011-000977-29; Spinifex Pharmaceuticals, Australia). C21 and EMA401 were taken forward into phase II and, in case of C21, phase III clinical trials and will be covered in detail in this review.

A. EMA401 – Neuropathic Pain

The first ever phase II clinical trial with an AT_2R-targeting drug tested the AT_2R antagonist EMA401 (developed by Spinifex Pharmaceuticals, Australia) in a multicenter, placebo-controlled, double-blind, randomized trial in patients with postherpetic neuralgia (ACTRN12611000822987) (Rice et al., 2014; Pulakat and Sumners, 2020). A total of 183 patients were enrolled, of which 92 received EMA401 (100 mg orally twice daily) and 91 placebo for 28 days. The primary endpoint was the change in mean pain intensity between baseline and the last week of dosing, which was determined by an 11-point numerical rating scale (NRS). Pain intensity was estimated and documented by patients daily. EMA401 was well tolerated and the primary endpoint was met by EMA401 treatment ($P = 0.0066$), although the difference to placebo on the NRS was only 0.7 points (2.3-point reduction in the EMA401 group vs. 1.6-point reduction in the placebo group) (Rice et al., 2014; Keppel Hesselink and Schatman, 2017). This successful phase II study led to the acquisition of all rights for EMA401 by Novartis, who subsequently initiated another two phase II trials, one for dose finding in patients with postherpetic neuralgia (EMPHENE trial; ClinicalTrials.gov Identifier: NCT03094195) and the other to test safety and efficacy in patients with painful diabetic neuropathy (EMPADINE trial; ClinicalTrials.gov Identifier: NCT03297294). Both trials had to be terminated prematurely because of liver toxicity of EMA401 in a preclinical, long-term toxicity study (Rice et al., 2021). At the time of termination, 129 of the 135 projected subjects of the first cohort in EMPHENE and 137 of the 400 projected subjects in EMPADINE had already been enrolled, and their study results were analyzed (Rice et al., 2021). The primary outcome for both studies was change in the weekly mean of the 24-hour average pain intensity score from baseline to week 12, using an 11-point NRS. There was no statistically significant effect of EMA401 on pain in both trials despite almost completed recruitment of the first EMPHENE cohort. Due to its toxicity, which was categorized as molecule specific but not as a class effect, clinical development of EMA401 has been terminated. However, other AT_2R-antagonistic follow up molecules are in the early stages of development (Isaksson et al., 2019; Guo et al., 2021). Nevertheless, since EMA401 may be a partial agonist, it is unknown whether the therapeutic efficacy observed in the first phase II trial was due to its agonistic or antagonistic properties. Thus, the development of "full" AT_2R antagonists may have to be reconsidered. Moreover, a very recent study demonstrated that EMA401 blockade of visceral hypersensitivity in animal models of irritable bowel syndrome was abolished by naloxone, implicating interference with opioid signaling (Nozu et al., 2021); thus, this is a potential additional mechanism to take into account when developing follow-up AT_2R ligands for analgesia.

B. C21 – COVID-19

In response to the SARS-CoV-2 pandemic and based on strong preclinical data as reviewed (Steckelings and Sumners, 2020), the AT_2R agonist C21 has been tested for safety and efficacy in a multicenter, randomized, double-blind, placebo-controlled, parallel-group phase II clinical trial in patients with COVID-19, the so called ATTRACT (Angiotensin II Type Two Receptor Agonist in COVID-19 Trial) trial (ClinicalTrials.gov Identifier: NCT04452435). A total of 106 patients were enrolled and randomized to receive either placebo (55 patients) or C21 (100 mg twice daily; 51 patients) orally for 7 days in addition to standard of care. Patients were followed up for an additional week. Eligibility criteria included hospitalization for COVID-19–associated respiratory symptoms but no mechanical ventilation at treatment start. The original primary endpoint, which was a change in C-reactive protein levels, became obsolete during the course of the trial because shortly before the start of patient enrollment, dexamethasone became the standard of care for COVID-19. Dexamethasone leads to a strong decrease in C-reactive protein levels, thus preventing a significant add-on effect by C21. Of the secondary endpoints, the number of subjects in need of oxygen supply was significantly reduced in C21-treated patients by the end of the follow up period (Tornling et al., 2021). The number of patients needing mechanical ventilation (one on C21 vs. four on placebo) and the number of deaths (one on C21 vs. three on placebo) were also reduced, but the number of events was too low to gain any statistically significant results. No major treatment-related side effects were reported.

In a follow-up study, high-resolution computer tomography obtained prior to, during, or up to 25 weeks after treatment with C21 or placebo from 33 patients (17 treated with C21, 16 receiving placebo) enrolled in ATTRACT were retrospectively evaluated in a blinded fashion for COVID-19 related pathological changes such as ground glass opacity, reticulation, band opacity, fibrosis, and consolidation [ATTRACT-2; ClinicalTrials.gov Identifier: NCT04878913). According to a

press release by Vicore Pharma, in patients treated with C21, 10.3% of the lungs displayed pathological anomalies versus 19.2% in patients in the placebo group (https://vicorepharma.com/investors/press-releases/press/?releaseID=214E6F22CE54D9E0).

Based on the ATTRACT results, the regulatory authorities have approved a pivotal randomized, double-blind, placebo-controlled, multinational, phase III trial (ATTRACT-3) that will include 600 adult patients hospitalized with COVID-19 and requiring oxygen support but not mechanical ventilation. Patients will be treated for 14 days with C21 (100 mg orally twice daily) or placebo followed by a 7-week follow-up period. The primary endpoint will be the "proportion of subjects discharged from hospital and free of supplemental oxygen" (ClinicalTrials.gov Identifier: NCT04880642). It is expected that this study will report in spring 2023.

C. C21 – Raynaud's Phenomenon in Systemic Scleroderma

Based on multiple preclinical studies showing a vasodilatory effect with AT_2R stimulation, Vicore Pharma initiated a phase II trial testing the effect of a single dose of C21 (200 mg orally) on cold-induced vasoconstriction in 12 female patients with systemic sclerosis Raynaud's phenomenon (mean age = 58.5 years) in a randomized, double-blind, placebo-controlled, crossover phase II trial (ClinicalTrials.gov Identifier: NCT04388176). As an important inclusion criterion, patients had to experience at least five Raynaud's phenomenon attacks per week. Raynaud's phenomenon was triggered by a standard cold challenge of their hands (15°C/15 minutes) 40 minutes after drug/placebo injection. The primary endpoint, which was an improvement of area under the curve (AUC) for rewarming of each finger after cold challenge as measured by infrared thermography, was not met, although there was a trend toward higher temperatures in the C21 group. However, patients treated with C21 had a higher maximal skin temperature 15 minutes after the cold challenge, indicating better perfusion compared with the placebo-treated subjects, and this effect was statistically significant (Herrick et al., 2022). Since by the end of the measurement period skin temperature had not reached a plateau but was still increasing, the time of measurement (40 minutes after drug application) may have been too early to capture the full effect.

D. C21 – Forearm Blood Flow

Another clinical trial with C21, which is based on the preclinical observation of AT_2R-induced vasodilation, is a phase I, open-label, single-center study in five healthy volunteers that will evaluate the effect of increasing doses of C21 (3, 10, 30, and 100 μg/min i.a. for 5 min/dose) on forearm blood flow (ClinicalTrials.gov Identifier: NCT05277922). With this study, the company will test a faster approach for elucidating optimal doses of future, new AT_2R-agonistic molecules (https://vicorepharma.com/investors/press-releases/press/?releaseID=5C37DC64436F081C).

E. C21 – Idiopathic Pulmonary Fibrosis

Prior to the COVID-19 pandemic idiopathic pulmonary fibrosis was the first indication for clinical development of the AT_2R agonist C21. Prioritization of a clinical trial in patients with COVID-19 plus the potentially increased risk of coronavirus infection in patients with idiopathic pulmonary fibrosis (a high-risk group) due to study participation delayed the onset of the phase II trial. The AIR trial is currently (Q3, 2022) still recruiting with the aim to enroll 60 patients to be treated with C21 (100 mg orally twice daily). The multicenter, open-label, single-arm (no placebo; comparison with historical controls) trial will examine the safety, efficacy, and pharmacokinetics of C21. Tolerability is listed as primary endpoint on ClinicalTrials.gov (ClinicalTrials.gov Identifier: NCT04533022). The sponsor's webpage also discloses a primary efficacy endpoint, which is a change in forced vital capacity at week 24 from baseline. The trial is expected to report in 2023. In February 2022, Vicore Pharma published data on their webpage about an interim analysis of the AIR study (https://vicorepharma.com/wp-content/uploads/2022/02/webcast-presentation-ipf-interim-220210.pdf). At that time, 21 of the projected 60 patients had been enrolled, of which 16 had completed the minimal treatment period of 24 weeks. The interim analysis revealed that patients experienced an average increase in mean forced vital capacity of 251 ml, whereas the historical control derived from four trials with almost 1000 patients saw a decline in forced vital capacity of 151 ml (King et al., 2014; Brown et al., 2019; Maher et al., 2019; Richeldi et al., 2020).

Key Points related to Section VII on *AT_2R Agonists and Antagonists in Drug Development Programs* are:

- In recent years, the AT_2R has been explored as a drug target in various clinical studies.
- AT_2R antagonists have been developed for the treatment of neuropathic pain, but the most advanced program was terminated due to molecule-specific toxicity.
- The most advanced drug development program for AT_2R agonists involves the small molecule drug C21 for use in COVID-19 (currently in phase III) and in idiopathic pulmonary fibrosis (currently in phase II).

VIII. Open Questions in AT_2R Research

Despite the wealth of research on the AT_2R over the last two decades, some questions on AT_2R biology are frequent topics of discussion and are still unresolved.

One such question concerns the true role of the AT_2 R during embryonic development. In this context, two AT_2R-related dogmata seem to contradict each other: on the one hand, AT_2Rs are highly expressed in embryonic tissue and are believed to play a role in embryo development, but on the other hand, mice with global AT_2R deficiency have hardly any developmental deficits. The question is: how can a receptor be important for embryo development but at the same time be dispensable? The common belief that AT_2Rs are generally and abundantly expressed in fetal tissue goes back to early studies in which AT_2R expression was determined by in situ receptor binding assays on sagittal frozen sections of fetal and neonatal rats using ^{125}I-Sar^1,Ile^8-angiotensin II (Jones et al., 1989; Grady et al., 1991). Looking at these data more closely, there are two important findings: 1) AT_2Rs are *not* ubiquitously expressed in high abundance in embryos, but high expression was restricted to undifferentiated mesenchyme of the intestine, skin, diaphragm, and tongue; and 2) AT_2R expression was strongest between E14 and E20 (i.e., toward the end of pregnancy when organogenesis has already been completed). However, there is one exception, which is organogenesis of the urogenital tract that is still ongoing on E20 and beyond (Grady et al., 1991). This may explain why the only defect in terms of organogenesis consistently described for AT_2R-KO mice is associated with urogenital malformations causing, for example, ureteral obstruction (Pope et al., 1998, 1999; Song et al., 2010). Apoptosis of mesenchymal cells surrounding the ureter and ureteral bud is an essential developmental process and at least partly mediated by AT_2Rs (Pope et al., 1998, 1999). Impaired disappearance of periureteral mesenchymal cells has indeed been described as a cause of ureteral obstruction in AT_2R-KO (Pope et al., 1998). In humans, there is an overproportional occurrence of the A-1332G AT_2 R transition in individuals with congenital anomalies of the kidney and urinary tract (CAKUT), which indicates that the AT_2R is also relevant for ureteral development in humans (Hohenfellner et al., 1999).

Another possible explanation for the seeming lack of developmental deficits in AT_2R-KO mice may be that such changes are subtle and only become apparent when mice are challenged (e.g., by prohypertensive, prodiabetic, proinflammatory, or profibrotic stimuli in respective disease models). For example, the increased BP rise in response to Ang II infusions (Hein et al., 1995a; Ichiki et al., 1995) may be a result of developmental deficits of aortic cells/VSMCs (Viswanathan et al., 1991), or the impaired glucose tolerance (Samuel et al., 2013; Quiroga et al., 2019) may be caused by incomplete pancreatic β-cell differentiation (Leung et al., 2014). Finally, the full phenotype in AT_2R-KO may not be attained due to induction of compensatory mechanisms such as an increase in activity of the ACE2/Ang-(1-7)/MAS axis of the RAS.

Surprisingly, the phenotypes of the two originally generated AT_2R-KO mouse strains—one from the group of Victor Dzau and Brian Kobilka (Hein et al., 1995a) and the other from the group of Tadashi Inagami (Ichiki et al., 1995)—were not identical, as already reviewed in Section V, the major difference probably being increased basal BP in the Inagami mice whereas baseline BP in the Dzau/Kobilka mice was normal. The cause of this difference is unknown but may either be attributed to slight differences in experimental conditions or to differences between the two mouse strains, which are in fact substantial. Although both strains were derived from an identical genomic clone (both isolated from a genomic DNA library of 129/SV mouse from Stratagene), they were backcrossed to different background strains: the Dzau/Kobilka mice to an FVB/N background (Hein et al., 1995a) and the Inagami mice to a C57Bl/6 background (Ichiki et al., 1995). Since it is generally known that different genetic backgrounds associated with different modifier or flanking genes can result in different phenotypes (Montagutelli, 2000; Eisener-Dorman et al., 2009), differences between the two AT_2R-KO strains are not really surprising. These differences may also be the reason for sometimes contradicting outcomes when AT_2R-KO mice of different background are attributed to disease models such as cardiac hypertrophy (Steckelings et al., 2010; Avila et al., 2011). It may also explain that in some disease models such as cardiac hypertrophy (Steckelings et al., 2010; Avila et al., 2011) or obesity (Yvan-Charvet et al., 2005; Chai et al., 2011; Samuel et al., 2013; Muñoz et al., 2017; Quiroga et al., 2019), inhibition of AT_2R actions by receptor antagonists elicits more consistent results than the use of AT_2R-deficient mice. Another conclusion from the controversy that may result from the use of AT_2R-KO mice is that the gold standard for studying AT_2R effects is the use of AT_2R agonists and not the "backward" approach of concluding on AT_2R effects from what is "lacking" when the receptor is antagonized or disrupted.

Another major open question and puzzling observation in AT_2R research is that stimulation of AT_2Rs can have pro- and antiapoptotic effects. For example, proapoptotic effects were described in embryonic mesenchymal cells (Kakuchi et al., 1995; Pope et al., 1998, 1999), PC12W and R3T3 cells (Yamada et al., 1996), cancer cells (Li et al., 2009; Zhao Y et al., 2015), and adult or embryonic VSMCs (Cui et al., 2001; Suzuki et al., 2002), whereas the AT_2R protected from apoptosis in various disease models such as MI (Kaschina et al., 2008), stroke (Schwengel et al., 2016), spinal cord injury (Namsolleck et al., 2013), or DN (Pandey and Gaikwad, 2017). In light of these opposing effects

of the AT_2R on apoptosis, the consequent question is what constitutes the "switch" between pro- and antiapoptotic properties. With regard to the AT_2R, there is no ultimate answer to this question. However, "life/death switches" have been described for several signaling mechanisms, which are strikingly also described to play a role in AT_2R-coupled signal transduction. For example, the serine/threonine phosphatase PP2A, which is a main component of AT_2R signaling as elaborated in this review, is involved in life/death decisions by modifying phosphorylation patterns of Akt, thus altering Akt downstream signaling (Andrabi et al., 2007). Pro- or antiapoptotic signaling of PP2A/AKT seems to depend on what other input (e.g., growth factors) the cell receives. Another example of a "life/death switch" is ERK1/2 MAPK activation, which in a complex interplay with other factors either inhibits or promotes apoptosis (Yue and López, 2020; Sugiura et al., 2021). The AT_2R was reported to be able to either induce or inhibit ERK1/2 MAPK depending on the conditions. One study reported both inhibition and activation of ERK1/2 in one and the same cell type, PC12W cells, observing AT_2R-mediated ERK1/2 MAPK activation in quiescent cells, whereas in NGF-stimulated PC12W, AT_2R-stimulation inhibited NGF-induced ERK1/2 MAPK activity (Stroth et al., 2000). These AT_2R ERK1/2 MAPK interactions indicate that the AT_2R not only signals through a mechanism that in itself is a "life/death switch" but that it can also activate or inhibit this mechanism (ERK1/2 MAPK), thus constituting a complex, still unresolved interplay with the potential to lead to pro- or antiapoptotic effects. Finally, HDAC1, which was only very recently identified to be involved in AT_2R-mediated regulation of apoptosis and proliferation (Peluso et al., 2022), promotes or inhibits proliferation depending on cell type and conditions (Dunaway and Pollock, 2022).

Collectively, the Janus-faced behavior of the AT_2R regarding life and death signals is not as unusual (or even unbelievable) as it seems but has also and independently been described for various signaling cascades that are involved in AT_2R signaling. Future experiments will have to provide proof that these known mechanisms are indeed responsible for an AT_2 R-associated "life/death switch" that, according to current, preliminary understanding, may elicit antiapoptotic, protective effects of the AT_2R in disease situations, whereas in healthy cells the AT_2R contributes to physiological cell turnover and differentiation through a mild proapoptotic effect.

IX. Conclusions and Future Perspectives

More than three decades of AT_2R research have in a vast majority of studies provided unquestionable evidence that this receptor is the central player of an endogenous protection, healing, and regeneration program that spans multiple organ systems. With few exceptions, AT_2R stimulation or knockdown has only minor effects in a healthy organism (see Section V), whereas its endogenous protective effects have been successfully applied in a multitude of disease models for therapeutic purposes (see Section VI).

Despite comprehensive knowledge of AT_2R activity in health and disease and the development of AT_2R ligands for clinical use (Section IV), AT_2R research is only now beginning to understand the structure and nature of this receptor, its activation mechanisms, and early upstream signaling. The recent crystallization of the AT_2R (see Section III.B) has provided some first insights in this field, but many more studies will be needed to fully understand this unusual receptor that does not seem to fit into the traditional GPCR categories.

A better understanding of AT_2R structure and its activation mechanisms will also illuminate the nature of its ligands, which, despite years of AT_2R research, seems to be more unclear than ever. In particular due to the lack of suitable functional screening assays, there is limited data to show which of the AT_2R ligands are full agonists and which are 'full' antagonists. Instead, the latest evidence suggests that most of the ligands, which are regarded as antagonists, are in fact partial agonists.

Our fragmented understanding of AT_2R ligands may negatively impact drug development programs since lead compounds that are chosen for their agonistic or antagonistic properties might not exert the maximum possible effect. Therefore, more reliable screening assays need to be developed, potentially based on new knowledge of the AT_2R structure, and all existing ligands must be reevaluated to identify new molecules with improved properties.

The final and most impactful questions that must be answered by future research concern the therapeutic efficacy of targeting the AT_2R. The effects of AT_2R in preclinical models are consistently protective but often rather weak. It remains unclear if these effects are strong enough for clinical use or if ligands could be optimized to get stronger effects. It is possible that the greatest potential of the AT_2R could be as part of a combination therapy. Finally, it remains unknown which of the indications that were successfully tested in animal disease models have the highest probability of successful translation into the clinical setting.

From an optimistic standpoint and in light of the most recent advances in AT_2R research, it seems realistic that most of the above questions will be answered within the next 5 to 10 years. However, undoubtedly new questions will arise and drive AT_2R research for many years to come.

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Authorship Contributions

Wrote or contributed to the writing of the manuscript: Steckelings, Widdop, Sturrock, Lubbe, Hussain, Kaschina, Unger, Hallberg, Carey, Sumners.

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