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International Union of Basic and Clinical Pharmacology. XCIX. Angiotensin Receptors: Interpreters of Pathophysiological Angiotensinergic Stimuli[S]

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ABBREVIATIONS: ACE, angiotensin converting enzyme; ADAM, A Disintegrin and Metalloproteinase; AGT, angiotensinogen; Ang(1-7), angiotensin(1-7, Asp^1-Arg^2-Val^3-Tyr^4-Ile^5-His^6-Pro^7); Ang, angiotensin; AngII, octapeptide angiotensin (Asp^1-Arg^2-Val^3-Tyr^4-Ile^5-His^6-Pro^7-Phe^8); AngIII, angiotensin 2-8 (Arg^2-Val^3-Tyr^4-Ile^5-His^6-Pro^7-Phe^8); AngIV, angiotensin 3-8 (Val^3-Tyr^4-Ile^5-His^6-Pro^7-Phe^8); ARB, AT_1 receptor blocker; AT_1 receptor, AngII type 1 receptor; AT_2 receptor, AngII type 2 receptor; AR234960, 1-[[(4*S*)-4-(3-fluorophenyl)-1-(2-methoxy-4-nitrophenyl)sulfonylpyrrolidin-3-yl]methyl]-4-pyridin-2-ylpiperazine; AR244555, 1′-but-3-enyl-5-chlorospiro[2H-indole-3,4′-piperidine]-1-yl)-(2,6-difluorophenyl)methanone; AT_3 receptor, AngII type 3 receptor; ATN, antiretroviral toxic neuropathy; ATRAP1, AT_1 receptor-associated protein; AVP, vasopressin; C21, Compound 21; CGP42112, nicotinic acid-Tyr-*N*-benzoxyl-carbonyl-Arg-Lys-His-Pro-Ile-OH; CHO, Chinese hamster ovary; CTGF, connective tissue growth factor; DAG, diacylglycerol; ECL, extracellular loop; eGFP, enhanced green fluorescent protein; EGFR, AT_1 receptor-associated protein (ATRAP1); EMA300, 5-[2,2-di(phenyl)acetyl]-4-[(4-methoxy-3-methylphenyl)methyl]-1,4,6,7-tetrahydroimidazo[4,5-*c*]pyridine-6-carboxylic acid; EMA401, (*S*)-2-(diphenylacetyl)-1,2,3,4-tetrahydro-6-methoxy-5-(phenylmethoxy)-3-isoquinolinecarboxylic acid; ERK, extracellular signal-regulated kinase; 3-D, three-dimensional; GPCR, G-protein–coupled receptor; GRK, G-protein–coupled receptor kinase; HF, heart failure; HGF, hepatocyte growth factor; ICL3, intracellular third loop; IL, interleukin; IP3, inositol triphosphates; IRAP, insulin regulated amino peptidase, also AngIV binding site; IRS, insulin receptor substrates; L-158809, 2-ethyl-5,7-dimethyl-3-[[4-[2-(2*H*-tetrazol-5-yl)phenyl]phenyl]methyl]imidazo[5,4-*b*] pyridine; LDL, low-density lipoprotein; MAPK, mitogen-activated protein kinase; MAS, putative Ang(1-7) receptor, also product of the *MAS* oncogene; NFκB, nuclear factor; NOS, nitric oxide synthase; PD123177, trifluoroacetate salt; PD123319, ditrifluoroacetate; PDGFR, platelet derived growth factor receptor; PKC, protein kinase C; PLC, phospholipase C; PLZF, transcription factor promyelocytic zinc finger protein; PRR, prorenin receptor; PTZ, pentylenetetrazol; RAS, renin-angiotensin system; ROS, reactive oxygen species; RTK, receptor tyrosine kinase; SHP, Src homology phosphatase; SHR, spontaneously hypertensive rats; SNP, single nucleotide polymorphism; TM, transmembrane; VEGF, vascular endothelial growth factor; VIF, vasoconstriction-inhibiting factor; VSMC, vascular smooth muscle cells.

Abstract*——The renin angiotensin system (RAS) produced hormone peptides regulate many vital body functions. Dysfunctional signaling by receptors for RAS peptides leads to pathologic states. Nearly half of humanity today would likely benefit from modern drugs targeting these receptors. The receptors for RAS peptides consist of three G-protein–coupled receptors—the angiotensin II type 1 receptor (AT_1 receptor), the angiotensin II type 2 receptor (AT_2 receptor), the MAS receptor—and a type II trans-membrane zinc protein—the candidate angiotensin IV receptor (AngIV binding site). The prorenin receptor is a relatively new contender for consideration, but is not included here because the role of prorenin receptor as an independent endocrine mediator is presently unclear. The full spectrum of biologic characteristics of these receptors is still evolving, but there is evidence establishing unique roles of each receptor in cardiovascular, hemodynamic, neurologic, renal, and endothelial functions, as well as in cell proliferation, survival, matrix-cell interaction, and inflammation. Therapeutic agents targeted to these receptors are either in active use in clinical intervention of major common diseases or under evaluation for repurposing in many other disorders. Broad-spectrum influence these receptors produce in complex pathophysiological context in our body highlights their role as precise interpreters of distinctive angiotensinergic peptide cues. This review article summarizes findings published in the last 15 years on the structure, pharmacology, signaling, physiology, and disease states related to angiotensin receptors. We also discuss the challenges the pharmacologist presently faces in formally accepting newer members as established angiotensin receptors and emphasize necessary future developments.

I. Introduction

The angiotensin receptor field has featured an enormous development since the last IUPHAR review, with >7255 peer review publications and reviews. Many of these belong to various facets of the type 1 angiotensin receptor followed by the type 2 receptor literature, which is closely followed by the upstart newcomer MAS receptor literature (Fig. 1). We have tried to identify all key papers and year-by-year break down on each of the four angiotensin receptors by carrying out systematic searches, initially using broad search terms and finally narrowing down to specific receptors. We recognize that computer searches do not fully cover all aspects of a particular receptor, but we have added selected references to more detailed reviews, which should be consulted for further information. To provide access to a wide range of specific information and the biologic activity data for each receptor detailed in this review, links are provided to IUPHAR/BPS receptor page for each receptor. Readers may also navigate to the IUPHAR/BPS Guide to Pharmacology website (www.guidetopharmacology.org).

A. History, Classic Components, and Functions of Renin-Angiotensin System

"Renin" was discovered in kidney extract as a hypertensive factor nearly 117 years ago by Tigerstedt and Bergman (1898). Hypertension in humans and

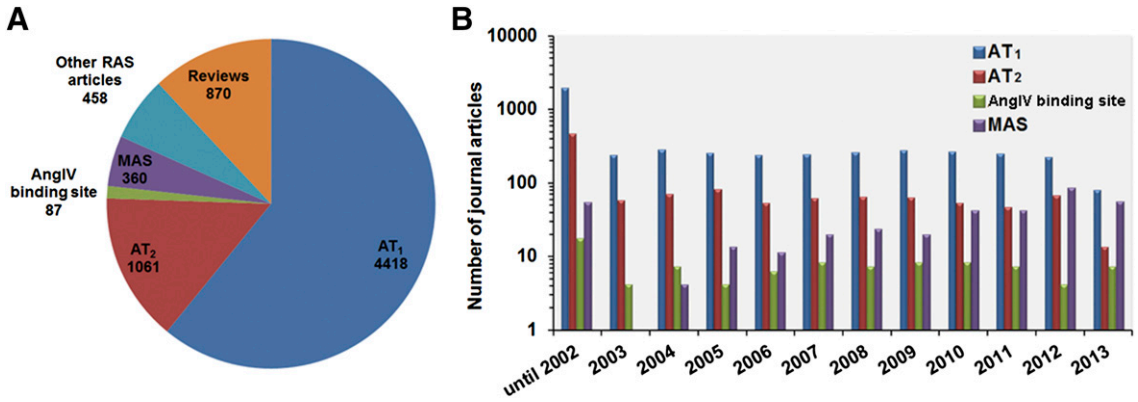

Fig. 1. Literature search and analysis of primary journal articles on RAS receptors. The journal articles published on RAS were automatically retrieved from PubMed with the search term "(((((RAS[Title/Abstract]) AND angiotensin, or AT_1 receptor, or AT_2 receptor, or AT_3 receptor, or AngIV binding site, or MAS1[Title/Abstract]) NOT medication adherence scale[Title/Abstract])". The extracted references list had a total of 9147 reviews, opinions, commentary, lectures, and primary journal articles through the end of year 2013. The results were manually curated to retain relevant, nonredundant references. The primary journal articles were separated from the reviews, interviews, lectures, or commentary articles. The pool of these 7255 primary journal articles were further analyzed to illustrate number of publications under each RAS receptor as in the pie chart (A). The bar graph represents publication for each receptor/year (B). Python and Bio python scripts were used to aid in the literature search and analysis.

animal models was described as a renovascular disease (Goldblatt et al., 1934). Page and Helmer (1940) isolated angiotensinogen, which they named as "renin activator" at that time, and they proceeded to isolate a vasoconstrictor substance "angiotonin" in the blood from live animals infused with renin. An identical vasoactive compound identified in Goldblatt hypertensive dog ischemic kidney by Braun-Menendez was named "hypertensin." The independently isolated pressor substance was later shown to be an octapeptide and not its decapeptide precursor (Skeggs et al., 1956; Bumpus et al., 1957; Elliott and Peart, 1957). Now the octapeptide bears the hybrid name angiotensin II (AngII) in honor of the original independent discovery of this important endocrine hormone with numerous actions beyond its hypertensive effects. The precursor AngI generated by renin action on angiotensinogen has no vasopressor activity. The sequence of human AngII is Asp^1-Arg^2-Val^3-Tyr^4-Ile^5-His^6-Pro^7-Phe^8. The quest for a peptide antagonist drug to control hypertension began with establishment of bioactivity of total chemically synthesized AngII (Bumpus et al., 1957; Rittel et al., 1957), which lead to an era of establishing the structure-activity relationship of angiotensin analogs in tissues (Khosla et al., 1974; Meyer et al., 1974; Peach and Levens, 1980).

The cascade of proteolytic steps leading to the formation of AngII in vivo and components of the renin angiotensin system (RAS) were characterized in later years (Fig. 2). RAS maintains normal blood pressure in vivo by regulating fluid volume and the vascular structure as well as integrity. AngII regulates blood volume through water-electrolyte balance (content) and also modulates cardiac output, vascular resistance (container). RAS is activated in response to decreased plasma sodium level and fluid volume, which stimulate juxtaglomerular cells in the kidneys to secrete renin. The enzyme renin cleaves angiotensinogen (AGT) released to circulation by liver to the decapeptide angiotensin I (AngI) in circulation. The octapeptide AngII is produced by the carboxypeptidase angiotensin converting enzyme (ACE1) predominantly located on endothelial cells. This constitutes the classic circulating RAS, an elaborate endocrine system that stimulates adrenal gland to release aldosterone to regulate fluid volume/electrolytes and nerve ends to release catecholamine to regulate vascular tone. It is a multifunctional hormone influencing many cellular processes, including cell growth, apoptosis, migration, inflammation, and fibrosis (Hunyady, 2009; Stegbauer and Coffman, 2011; Horiuchi et al., 2012).

B. Tissue Renin-Angiotensin System

Apart from being a circulating hormone, locally produced AngII in most tissues functions as a paracrine and autocrine hormone (Dzau and Gibbons, 1987; Griffin et al., 1991; Weber et al., 1995). In this mode, the tissue RAS regulates long-term and chronic responses to locally produced AngII in tissues, including the brain, heart, kidneys, pancreas, vasculature, and adipose tissue. Importantly, tissue RAS functions independently of circulating RAS, providing critical paracrine or autocrine control in pathophysiological conditions, including hypertension, inflammation, thrombosis, atherosclerosis, diabetes, end-stage renal disease, coronary artery disease, cardiovascular hypertrophy, and heart failure (HF) (Lijnen and Petrov, 1999; Kim and Iwao, 2000; Lavoie and Sigmund, 2003; Mehta and Griendling, 2007).

C. Intracellular Renin-Angiotensin System and Alternative Pathways Generating Angiotensin Peptides

Cardiac and vascular smooth muscle cells and fibroblasts have been reported to harbor an intracellular AngII production pathway sometimes also referred as an "intracrine system" (Kumar et al., 2009). Often the

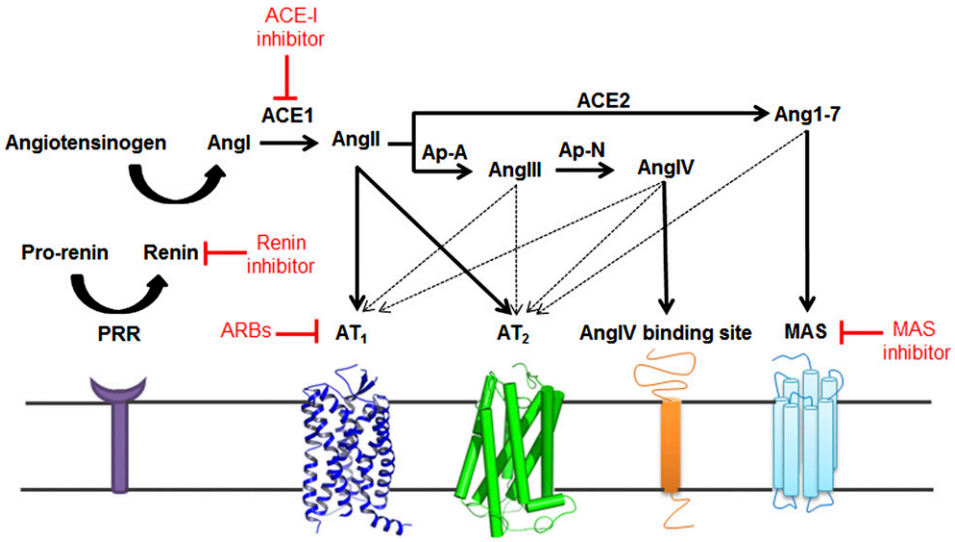

Fig. 2. A current view of enzymatic pathways generating hormonal peptides of RAS and corresponding cell surface receptors. Surrogate enzymes, such as mast cell chymase, neutral endopeptidase, tonin, cathepsin D, kalikrine, and aminopeptidases are also known to participate in angiotensin metabolism in different contexts. Some authors contend inclusion of these enzymes as components of RAS. Inhibitors of different components of RAS are shown in red.

major components of RAS, renin, AGT, and ACE do not coexist in most cells to fully support an intracellular RAS paradigm. However, alternative enzymes, such as cathepsins and chymases may produce functional AngII intracellularly (Kumar et al., 2008; Kumar and Boim, 2009). Depending on the cell type, cathepsin D can participate in the first step of intracellular AGT cleavage usually catalyzed by renin and the second step by chymase, not ACE (Wasse et al., 2012). Obviously, ACE inhibitors do not inhibit the intracellular RAS. Similarly, intracellular AngII actions are not prevented by Ang receptor blockers (Baker et al., 2004; Baker and Kumar, 2006; Singh et al., 2007). Importance of intracellular RAS concept in pathophysiology of disease is extensively described in several reports (Carey, 2012; Cook and Re, 2012; Ellis et al., 2012; Gwathmey et al., 2012; Kumar et al., 2012). However, controversy exists because it is recognized that extracellular AngII may be internalized by receptor-mediated processes and serve as the source of intracellular AngII (Re and Cook, 2011).

ACE-independent, alternative pathways generating angiotensin peptides AngI, AngII, AngIII, Ang(1-7), and AngIV (Fig. 2) have been described. These are intricate, highly efficient endogenous pathways in cardiac and smooth muscle cells, renal tubular, mesenchymal, as well as inflammatory cells infiltrating various tissues. Contribution of alternative pathways involving chymases, tonins, neutral endopeptidases, and aminopeptidases can significantly vary relative to classic RAS in a pathologic setting (Carey and Siragy, 2003; Chua et al., 2010; Wasse et al., 2012; Naffah-Mazzacoratti et al., 2014; Su, 2014).

D. Newer Components and Functional Axis of Renin-Angiotensin System

The function of classic RAS was thought to be rather simple, to regulate formation of AngII as shown in Fig. 2. However, clinical application of RAS blockers demonstrated that these agents do not uniformly control circulating and tissue AngII levels. The concentration of AngII may often increase above pretreatment levels under therapy, which suggests that the beneficial effects are not due to blocking AngII production. Perhaps AngII is further metabolized to produce the beneficial effects. Paradigm shift discovery of angiotensin(1-7) [Ang(1-7)] as the antagonist of physiologic actions mediated by AngII and the discovery of angiotensin-converting enzyme 2 (ACE2) that produces Ang(1-7) in vivo has provided basis for this. More than a decade of research has unraveled a second arm of RAS (Fig. 2) that is composed of the monocarboxypeptidase ACE2, producing Ang(1-7) through hydrolysis of AngI or AngII, and counterbalancing the pressor arm constituted by renin-angiotensinogen-AngI-ACE, leading to production of AngII (Ahmad et al., 2011). This arm of RAS is considered the counter-regulatory axis. Both animal and clinical studies have emerged to define a role for ACE2 in regulating the progression of cardiovascular disease and pulmonary arterial hypertension. The activation of pulmonary ACE2 could serve as a novel therapeutic target in vivo (Bradford et al., 2010; Jiang et al., 2014). The ACE2/Ang(1-7) axis seems to be involved in many physiologic and pathophysiological processes in several systems and organs, especially by opposing the detrimental effects of inappropriate overactivation of the ACE/AngII axis (Passos-Silva et al., 2013).

Blockade of RAS with drugs targeting various components disturbs feedback control of AngII levels by highly regulated release of renin. As a result, the increase in plasma renin causes AngII and aldosterone levels to surge. In addition, the levels of prorenin also increase, a key observation that led to the discovery of the prorenin receptor (Fig. 2) (Nguyen et al., 2002; Batenburg et al., 2004). Higher levels of prorenin than renin are seen in blood plasma during hypertension and diabetes. The prorenin receptor (PRR) is a ubiquitously expressed 350-amino acid protein, previously described as Na/H^+ ATPase. PRR can bind both renin and prorenin and induce nonproteolytic prorenin activation and generation of AngI. PRR-mediated activation is a distinct mechanism from classic proteolytic activation of prorenin by an unidentified enzyme restricted to kidney. Renin inhibitors do not block the PRR activation of prorenin. PRR expression levels are high in brain regions, which could be particularly relevant because the expression of classic RAS components is low. The PRR-mediated oxidative stress in central nervous system is caused by increased reactive oxygen produced by overexpressed NOX2 and NOX4. This regulation is independent of AngII formation and involves ERK-PI3K/Akt signals directly induced by PRR binding of prorenin (Peng et al., 2013). Neuron-specific PRR gene knockout prevents development of deoxycorticosterone acetate salt-induced hypertension and brain AngII production (Li et al., 2014). PRR may thus be a critical membrane-bound prorenin receptor with signaling ability that regulates physiology. The PRR thus may contribute to angiotensin surges as well as directly activate transmembrane signaling independently. The discovery of PRR has renewed interest in the physiology of the RAS (Guang et al., 2012).

E. Angiotensin Receptors Interpret Functions of Renin-Angiotensin System

The concept of a plasma membrane receptor in target cells emerged based on specific recognition of AngII and stimulation of response such as adrenal steroidogenesis, nerve catecholamine release, and aortic contraction (Peach, 1977; Devynck et al., 1978). In the 1980s, pharmacological nonpeptide antagonists Dup753 and PD123177 were instrumental in demonstrating two types of AngII receptors in tissues, which were indistinguishable by peptide analogs (Chiu et al., 1989; Whitebread et al., 1989; Speth and Kim, 1990). Other angiotensin-derived metabolites such as Ang1-7, or Ang3-8 (AngIV) have all been shown to have distinct biologic activities (Peach, 1977; Ferrario et al., 1991; Wright et al., 1995; Iyer et al., 1998). Metabolism of AngII via aminopeptidases A and N generates AngIV (Padia and Carey, 2013). Effector organs responded differently to AngII and its metabolites based on the presence of distinctly different receptors, their abundance in target tissue, their selectivity for the agonists, their structure-activity relationships, signal transduction specificity, and regulation, desensitization and trafficking. G-protein–coupled receptors (GPCR) that elicit a response to AngII and Ang(2-8) (AngIII) are the angiotensin II type 1 (AT_1 receptor) and angiotensin II type 2 (AT_2 receptor), which are extensively studied (Teerlink, 1996; de Gasparo et al., 2000). MAS, an orphan GPCR, has been functionally and pharmacologically linked to Ang(1-7) and is a strong candidate target for mediating the ACE2-Ang(1-7) axis of RAS. A class I membrane protease, insulin regulated amino peptidase (IRAP), is a strong candidate for AngIV receptor with distinctly different function compared with the GPCRs of RAS.

The decapeptide angiotensin I (AngI) serves as the reference for residue numbering of all angiotensin metabolites, as adopted by the International Society for Hypertension, The American Heart Association, and the World Health Organization (Dzau and Gibbons, 1987). Along this convention, the receptors for specific angiotensin metabolites are named Ang receptors (currently MAS is an exception). The classification of angiotensin receptors proposed in 1991 was updated in 2000 and 2014 (Bumpus et al., 1991; de Gasparo et al., 2000; Karnik et al., 2014).

The criteria used for inclusion of Ang receptors include the affinity and selectivity of agonists and antagonists for the receptor followed by transduction criteria, which is dependent on agonist-specific receptor-effector coupling. Finally, the gene and receptor sequence and the three-dimensional structure, if available, are the structural criteria. Signal coupling mechanisms and physiologic tissue responses in some instances have been major influencing factors on differentiating receptor types. Gene and receptor structures, cloning and heterologous expression combined with pharmacological validation were used in defining different receptors, which respond to specific metabolite fragment angiotensins, ultimately validating the structural basis for classification. Thus, a combination of several criteria was used in defining true receptor types. However, all criteria not met with equitable rigor for different Ang receptors remain a limitation.

Principles used for angiotensin receptor nomenclature were documented previously (de Gasparo et al., 2000). The receptors are AT_1–AT_3. Additional subdivisions (e.g., AT_{1b}) and species (hAT_1 or mAT_2) are identified for pharmacologically defined receptor subtypes in different species. The Ang receptors AT_1 and AT_2 fully meet classification criteria, with IUPHAR Receptor Code of 2.1.Ang.01.000.00.00 and 2.1 Ang.02.000.00.00 (Humphrey and Barnard, 1998). They are seven transmembrane domains rhodopsin subclass GPCRs. The human genome contains single genes *AGTR1* and *AGTR2*, which encode AT_1 and AT_2 receptors, respectively. The name AT_3 receptor was originally assigned based on AngII binding described in the

Neuro-2a mouse neuroblastoma cell line. AngII binding to sites in these cells was not blocked by the AT_1-specific losartan, and the AT_2-specific PD123319 and was not affected by GTP analogs (Chaki and Inagami, 1992b). Without the knowledge of the complete human genome at that time, cloning efforts directed at finding additional angiotensin receptor genes in humans lead to the realization of segmental duplications and rearrangements in unrelated loci but not a second AT_1 receptor gene (Iafrate et al., 2004). Consequently, an *AGTR3* gene is also not assigned in the completed human genome despite molecular identification of a putative cDNA clone for the proposed AT_3 receptor. The existence of a genuine AT_3 receptor is not confirmed at this time.

Additional angiotensin receptors (AngIV binding site and MAS) are proposed based on ligand interaction and physiologic functional criteria. The AngIV binding site may play a significant role in the central nervous system, kidney, and vasculature. The cloned AngIV binding site is an allosteric transmembrane zinc protease. The selectivity of endogenous AngIV for this receptor is not clear, and the physiologic characteristics evaluated in gene knockout and overexpression mouse models have not yielded a conclusive unifying picture. The signal transduction mechanisms of the AngIV binding site are unknown. Also, other functional surrogate AngIV binding sites proposed have generated some confusion. This transmembrane protease is thus a strong candidate for consideration as AngIV binding site. MAS is an exception to the Ang receptor nomenclature system, because it is an orphan GPCR capable of signal transduction in response to several unrelated peptides and angiotensin metabolites. The confusing pharmacology documented for this receptor, including the possibility of multiple endogenous ligands and confusing transduction modalities, is the reason for not elevating it to Ang(1-7) receptor. Multiple endogenous ligands are not unusual but AngIV binding site and MAS as physiologic entities do not rise to the level of acceptance as specific Ang receptors, because pharmacology and signaling are not rigorously established.

II. The Angiotensin II Type 1 Receptor

The lion's share of literature on RAS receptors belongs to the AT_1 receptor as reflected by 4418 peer reviewed articles mined in our search (Fig. 1; Unal et al., 2014, http://www.guidetopharmacology.org/GRAC/ObjectDisplayForward?objectId=34). Major effects on renal and cardiovascular physiology attributed to AngII are mediated through the AT_1 receptor (Dinh et al., 2001). Chronic activation of the AT_1 receptor can lead to disease states including hypertension, cardiac arrhythmia, stroke, diabetic nephropathy, and metabolic disorders (Audoly et al., 2000; de Gasparo et al., 2000; Zaman et al., 2002; Thomas and Mendelsohn, 2003), which are effectively treated using AT_1 receptor blockers (Zaman et al., 2002; Billet et al., 2008; Akazawa et al., 2013; Michel et al., 2013; Seva Pessoa et al., 2013). The cDNAs for the AT_1 receptor were cloned from rat smooth muscle and bovine adrenal gland (Murphy et al., 1991; Sasaki et al., 1991). The AT_1 receptor of human, mouse, rabbit, pig, dog, turkey, and frog were characterized later. Human genome harbors a single gene, *AGTR1*, which mapped to chromosome 3q21-3q25. Rat and mouse genome harbors two distinct AT_1 receptor genes, *Agtr1a* (chromosomes 17 and 13, respectively) and *Agtr1b* (chromosomes 2 and 3, respectively). The AT_{1a} receptor and AT_{1b} receptor share 95% amino acid sequence homology. Noncoding regions of their genes are strikingly different, suggesting possible differences in tissue-specific expression and regulation of AT_{1a} receptor and AT_{1b} receptor (de Gasparo et al., 2000). Indeed, both receptor subtypes are pharmacologically and functionally identical, but they differ in tissue distribution and transcriptional regulation. The AT_{1a} receptor is well expressed in most cardiovascular tissues and is the principal regulator of blood pressure. The AT_{1b} receptor expression is limited to endocrine tissues such as the adrenal and pituitary glands (Kakar et al., 1992; Ito et al., 1995; Sugaya et al., 1995; Chen et al., 1997). The *AGTR1* gene contains five exons and four introns, out of which exon 5 codes for the hAT_1 receptor polypeptide (Sasaki et al., 1991). Human *AGTR1* splice variant mRNAs containing exon 2 are poorly translated. In contrast, mRNA splice variants, which harbor exon 3, produce a longer hAT_1 receptor isoform with additional 32 N-terminal amino acids. The long-form of hAT_1 receptor displayed a threefold diminished affinity for AngII. Splice variants of hAT_1 receptor may ultimately affect AngII responsiveness in a given tissue (Warnecke et al., 1999; Elton and Martin, 2003). The early characterization of AT_1 receptor pharmacology and signal transduction property is comprehensively reviewed by de Gasparo et al. (2000).

A. Structure-Function

The genomic DNA of all mammalian species contains an open reading frame for 359 amino acid residues, yielding ~41 kDa calculated molecular weight for AT_1 receptor (Guo et al., 2001). AT_1 receptor belongs to the rhodopsin branch of the GPCR superfamily. The extracellular domain consists of the N terminus and the extracellular loops (ECLs) and contains three *N*-glycosylation sites. Four cysteine residues in the extracellular domain of the AT_1 receptor are predicted to form two disulfide bonds (Fig. 3A), which are prone to inactivation by dithiothreitol and other reducing agents (Warnecke et al., 1999). The three intracellular loops form the G protein activation domain. The cytoplasmic C-terminal tail contains phosphorylation sites for serine/threonine kinases, including protein kinase C (PKC) and GPCR kinases. Functionally, the AT_1 receptor is primarily coupled through the G_q protein to

phospholipases C, A2, and D. Similar to other prototypical GPCRs, agonist activation leads to desensitization and internalization of AT_1 receptor.

The three-dimensional structure at room temperature with 2.9-Å resolution was determined for hAT_1 receptor bound to the experimental antihypertensive agent ZD7155 using a novel, X-ray–free approach through collaborative efforts (Zhang et al., 2015). The three-dimensional structure confirms a canonical seven-transmembrane (TM) α-helical architecture with precise boundaries for N terminus, three extracellular loops (ECL1-3), three intracellular loops (ICL1-3), an amphipathic helix VIII, and the C terminus (Figs. 2 and 3). The AT_1 receptor three-dimensional structure is most similar to chemokine and opioid receptors. The predicted disulfide bonds, Cys18–Cys274 connecting the N terminus and ECL3, and Cys101–Cys180 connecting helix III and ECL2 are confirmed. The ECL2 of AT_1 receptor exhibits a β-hairpin secondary structure as in other peptide GPCRs (Fig. 3B). Intriguingly, ECL2 of AT_1 receptor serves as an epitope for the agonistic autoantibodies in preeclampsia and malignant hypertension (Unal et al., 2012; Xia and Kellems, 2013). The conserved DRY motif in helix III and the NPxxY motif in helix VII of AT_1 receptor were proposed to participate in receptor activation (Oliveira et al., 2007).

An agonist bound AT_1 receptor structure is unavailable at this time. However extensive site-directed mutagenesis studies combined with biochemical and pharmacological experiments on mammalian AT_1 receptor have provided insights into AngII binding, mechanism of receptor activation, G-protein interaction, as well as regulation by desensitization and internalization (Hjorth et al., 1994; Marie et al., 1994; Schambye et al., 1994; Noda et al., 1995a; Yamano et al., 1995; Hunyady et al., 1996, 1998; Karnik et al., 1996; Monnot et al., 1996; Balmforth et al., 1997; Groblewski et al., 1997; Inoue et al., 1997; Han et al., 1998).

The current view is that AngII binding to the AT_1 receptor involves two salt bridges, between the α-carboxyl group of AngII and Lys^{199} of AT_1 receptor and between the guanidinium group of Arg-2 in AngII and Asp^{281} in the receptor (Yamano et al., 1992; Feng et al., 1995; Noda et al., 1995a; Miura et al., 2003a). Ligand-crosslinking approach showed interactions between Phe-8 of AngII and Phe^{293} and Asn^{294} in AT_1 receptor in TM domain (Perodin et al., 2002). Another interaction observed by crosslinking between Val-3 of AngII and Ile^{172} in AT_1 receptor is consistent with an ion pair suggested between Asp-1 in AngII and His^{183} by mutagenesis data (Feng et al., 1995; Boucard et al., 2000). Arg^{23} in N terminus of AT_1 receptor may be essential for binding AngII (Santos et al., 2004a). Thus, the hydrophobic carboxyl terminal region of AngII appears to enter the TM-domain core of the receptor and amino terminal region of AngII appears to interact with extracellular region of the AT_1 receptor. A two-step model for binding of AngII to AT_1 receptor has been proposed (Le et al., 2002; Feng et al., 2005). Boucard et al. (2000) suggested an extended conformation of AT_1 receptor-bound AngII (Perodin et al., 2002; Fillion et al., 2013). The methionine proximity mapping approach they used identified details of the residues lining the AngII binding

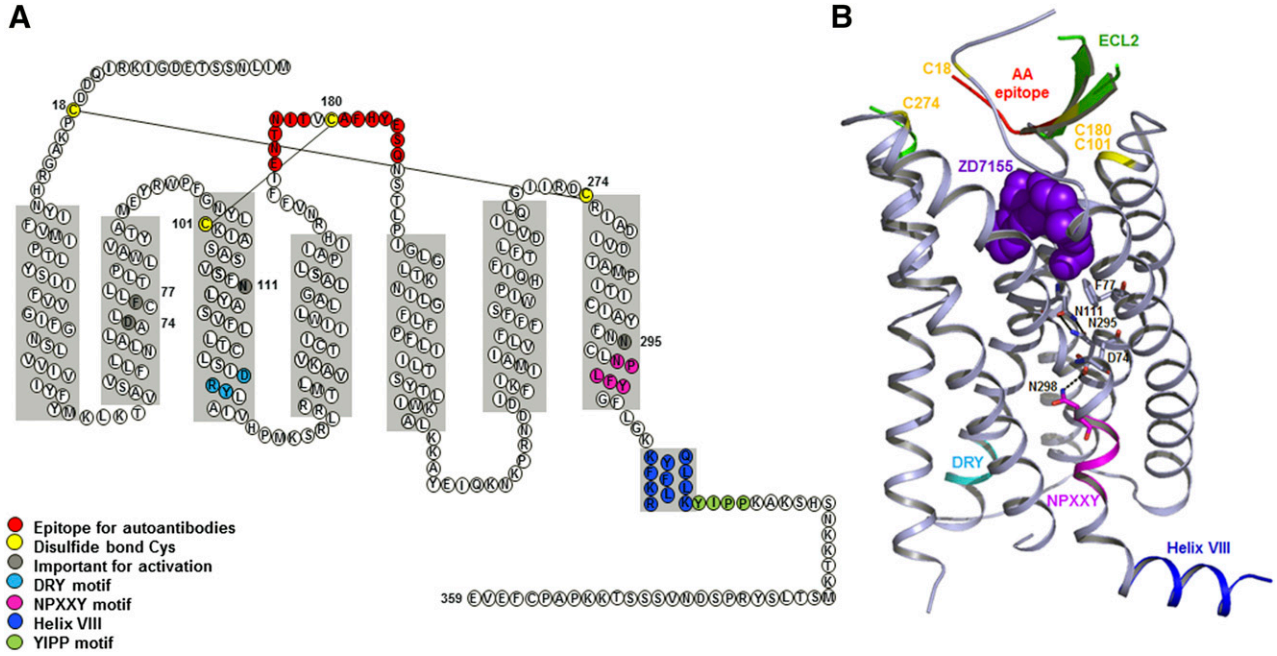

Fig. 3. Structure of human AT_1 receptor. (A) Secondary structure of human AT_1 receptor with precise depiction of α-helical borders and sequence motifs. (B). Overall three-dimensional structure of AT_1 receptor-antagonist complex, detailing AT_1 receptor with different motifs and antagonist. The critical hydrogen bond between Asn^{111} and Asn^{295} is indicated. Close proximity of residues such as Asp^{74} and Asn^{298} is suggestive of Na-ion coordination site. Sodium concentration may modulate receptor activation by agonists.

pocket of AT_1 receptor. These residues include Phe^{77}, Leu^{112}, Tyr^{113}, Phe^{249}, Trp^{253}, His^{256}, Thr^{260}, and Phe^{293}, Asn^{294}, Asn^{295}, Cys^{296}, and Leu^{297} (Correa et al., 2002; Clement et al., 2005, 2006, 2009). Almost all of these residues are within 3-Å distance of the bound antagonist in the crystal structure, which substantiates the idea that the agonist, AngII, and AT_1 receptor antagonists share an overlapping binding pocket of the AT_1 receptor.

Intrinsic stability prevents spontaneous activation of AT_1 receptor, and agonists overcome this stability barrier during activation. AngII is proposed to mediate activation of AT_1 receptor through stacking interactions between Phe-8 of AngII and His^{256} (Noda et al., 1995a) and between Tyr-4 of AngII and Asn^{111} in AT_1 receptor (Noda et al., 1996; Miura et al., 1999). Interhelical interaction between Asn^{111} and Tyr^{292} in the inactive receptor is proposed to be disrupted upon AngII binding, allowing Tyr^{292} to interact with Asp^{74} (Joseph et al., 1995). Smaller residue substitutions for Asn^{111} have been shown to induce constitutively activated AT_1 receptor conformation (Groblewski et al., 1997; Feng et al., 1998; Miura et al., 1999). The three-dimensional structure suggests that Asn^{111} hydrogen bonds with Asn^{295}, and disruption of this interaction may cause constitutive activation of AT_1 receptor. Several other TM mutations that potentially disrupt intrinsic stability in AT_1 receptor are reported to produce constitutive activation, including Asn^{295}, Asp^{125}, Phe^{77}, Leu^{112}, Leu^{118}, Leu^{195}, Ile^{245}, and Leu^{305} (Parnot et al., 2000). Nikiforovich et al. (2005) showed that AT_1 receptor constitutive activity is also elicited when bulkier residues were introduced in TMIII without mutating Asn^{111}. They proposed that by mutating Asn^{111} and adjacent bulky residues, a cascade of conformational perturbations activated the receptor.

Activation of AT_1 receptor is shown to be associated with translation and rotation of TM helices, including TMII, TMIII, TMV, TMVI, and TMVII by two independent methods, reporter cysteine accessibility mapping and methionine proximity analysis (Miura and Karnik, 2002; Boucard et al., 2003; Miura et al., 2003b; Martin et al., 2004, 2007; Domazet et al., 2009a,b; Arsenault et al., 2010a). The residues Met^{30} and Thr^{33} in TMI, as well as Arg^{167} and Val^{169} in ECL2, are shown to interact with AngII, but these regions undergo very little movement during AT_1 receptor activation (Yan et al., 2010). Only subtle structural changes were identified between the AT_1 receptor and its constitutively active form (Clement et al., 2006). Binding of structurally different ligands could produce different active receptor conformations (Miura et al., 2012).

ECL2 is a critical determinant of ligand-specific conformational changes resulting in activation or inhibition of the AT_1 receptor (Unal et al., 2010). Unal et al. (2010) suggested that ECL2 might assume a lid conformation induced upon binding both agonist and antagonist but exposing distinct residues around the highly conserved disulfide bond between Cys^{180} and Cys^{101}. A role in coupling the movements of TM helices to the ECL2 during receptor activation was suggested (Ohyama et al., 1995; Karnik et al., 2003; Unal et al., 2010). Interestingly, the ECL2 assumed a lid conformation in the gain-of-function mutant N111G-AT_1 receptor without agonist stimulation (Unal et al., 2013). In contrast, AngII did not induce a lid conformation in the loss-of-function mutant D281A, which is consistent with poor AngII binding in this mutant. However, a lid conformation was regained when an AngII analog that is specific for D281A mutant bound the mutant receptor. On the basis of these results an emerging paradigm of domain coupling facilitated by long-range interactions was proposed. Domain coupling has been postulated as a mechanism for explaining a range of GPCR conformations with different functional consequences regulated not only by classic agonists and antagonists, but also by a variety of nonclassic factors including receptor-interacting proteins, dimerization, and activation by autoantibody (Unal and Karnik, 2012).

Mutational analysis has shown that Tyr^{127} to Met^{134} region (ICL2) and the Ile^{238} to Phe^{239} region (ICL3) are important for $G_{q/11}$ activation by the AT_1 receptor (Miura et al., 2000; Zhang et al., 2000; Gaborik et al., 2003). Critical single residues in the intracellular region of the AT_1 receptor required for G-protein activation include Arg^{126} (TMIII), Tyr^{215} (TM V), Leu^{222} (ICL 3), Tyr^{312}, Phe^{313}, and Leu^{314} (TMVIII) (Oliveira et al., 2007). The carboxyl tail of AT_1 receptor regulates AT_1 receptor desensitization and internalization (Hunyady and Catt, 2006; Smith and Luttrell, 2006) and it also directly associates with downstream effectors and plays crucial roles in signal transduction, internalization, and other functions of the receptor (Cruse et al., 1992; Ali et al., 1997; Venema et al., 1998a; Horiuchi et al., 2012). A stretch of C-terminal residues 305–320 in AT_1 receptor is known as the helix VIII (Huynh et al., 2009). Multiple lines of evidence suggest contribution of helix VIII to AT_1 receptor expression and trafficking, G-protein coupling and activation, receptor internalization, dimerization, and signaling by mediating protein-protein and protein-lipid interactions. The Tyr-Ile-Pro-Pro (YIPP, amino acids 319–322 in Fig. 3A) motif within helix VIII is essential for activation of the Jak-STAT pathway, PLC-gamma phosphorylation, and the AngII-induced intracellular calcium transients (Venema et al., 1998a).

B. Pharmacology

Nonpeptide AT_1 receptor antagonists, referred to as AT_1 receptor blocking (ARB) drugs, are a cornerstone of therapy for not only lowering blood pressure and protecting renal damage, but are under consideration for a variety of other human disease conditions. At least eight different ARBs are clinically available at this time. They are losartan, candesartan, olmesartan, telmisartan, eprosartan, irbesartan, valsartan, and

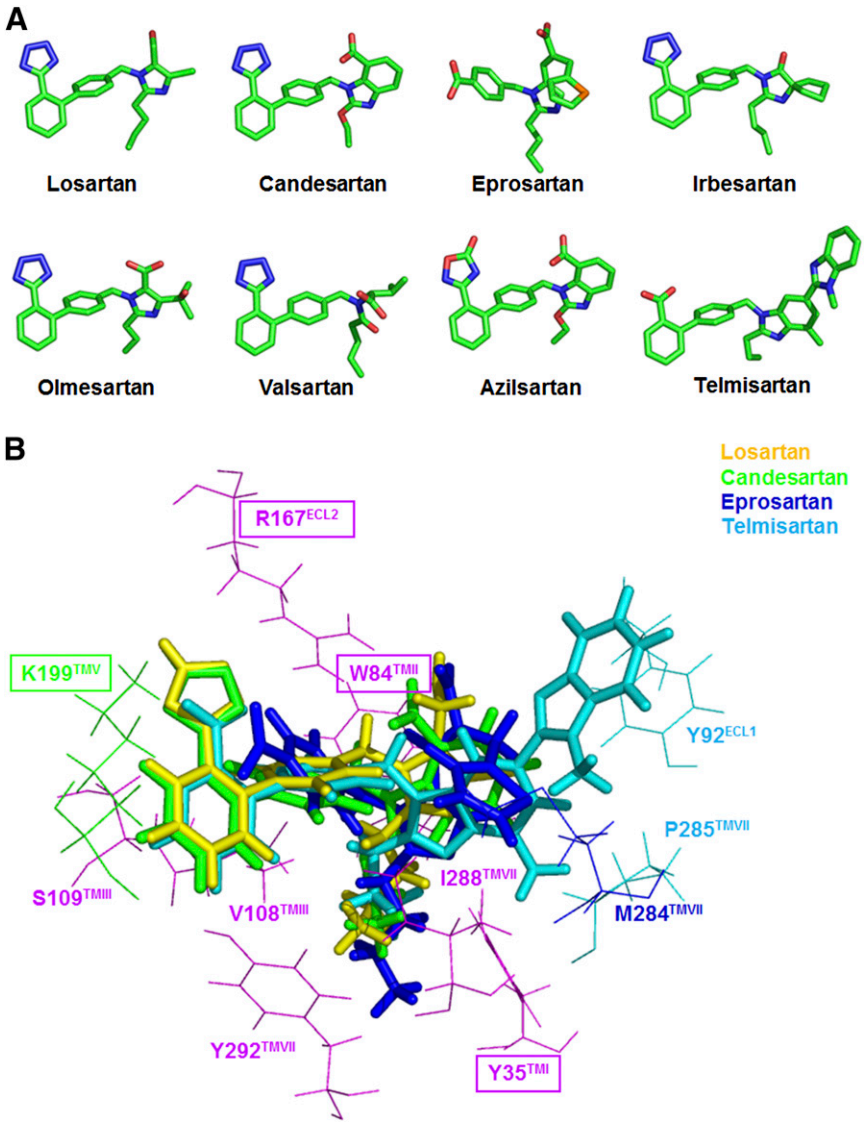

Fig. 4. (A) Three-dimensional structural details of ARBs in clinical use; and (B) interaction with human AT_1 receptor. Differences in the efficacy of different ARBs may be due to small differences in interaction of shown ARBs with residues such as Tyr^{92}, Trp^{182}, Lys^{199}, Met^{284}, and P^{285}.

azilsartan (Fig. 4A). ARB development efforts critically focus on binding to AT_1 receptor and oral bioavailability, insurmountability, inverse agonism (Kohara et al., 1996; Miura et al., 2006; Fujino et al., 2010). Candesartan cilexitil, losartan, and olmesartan medoxomil are prodrugs of candesartan, EXP3174, and olmesartan, respectively (Schmidt and Schieffer, 2003). Most ARBs do not cross the blood-brain barrier but central effects may be observed due to prolonged treatment or under pathologic conditions where the blood-brain barrier may become more permissive. Telmisartan is reported to cross the blood-brain barrier, but losartan, candesartan, olmesartan, azilsartan, and valsartan are reported to poorly cross the blood-brain barrier. Effects on the central nervous system are reported in preclinical studies for several ARBs, which may afford beneficial effects in Alzheimer's disease in addition to blood pressure lowering (Duron and Hanon, 2010). Physiologic responses to in vivo antagonism of the AT_1 receptor have been quantified by measurement of renin release (Munafo et al., 1992; Muller et al., 1994; Maillard et al., 2002), aldosterone release (Ogihara et al., 1995), and plasma NO production (Gossmann et al., 2001) in response to AngII in humans.

1. Pharmacophore Structure-Activity Relationship. Structurally, the biphenyl tetrazole seen in losartan, EXP3174, ZD7155, and candesartan or an acidic moiety as seen in telmisartan, 5-oxo-1,2,4-oxadiazole, as in azilsartan change lipophilicity of ARBs (Vyas and Ghate, 2010). Eprosartan has a different structure, biphenyltetrazole replaced with benzoic acid. Consequently, ARBs are designed to exploit hydrophobic interaction of the phenyl rings with transmembrane core of AT_1 receptor and bind basic residues in AT_1 receptor forming ionic interactions with the acidic moieties. Losartan has an imidazole with Cl at the other end of the molecule,

and its metabolite, EXP3174, has a COOH substituent. Sartans present a greater variety of structures at this end that probably account for some of their functional differences. Olmesartan and losartan are closely related. Irbesartan has a cyclopentyl ring incorporated in place of the Cl. A benzimidazole is substituted in candesartan and azilsartan; two benzimidazoles are attached in telmisartan to produce an unusual structure for this ARB. The unique structure of valsartan lacks a nitrogen containing heterocycle. The imidazole ring in eprosartan has a large alkyl chain substituent. The difference in structure is expected to cause the ARBs to bind in slightly different ways.

In the crystal structure of AT_1 receptor bound to ZD7155, the ligand-binding pocket shows exactly how ARBs might interact with AT_1 receptor (Fig. 3). The AT_1 receptor residues, mainly from helices I, II, III, VII, and ECL2, directly interact with the ligand. Side chains of $Arg^{167(ECL2)}$ and $Tyr^{35(TM1)}$ form ionic and polar interactions with ZD7155. The acidic tetrazole moiety closely interacts with the basic $Arg^{167(ECL2)}$, and this residue may be primarily responsible for ARB-binding affinity and selectivity for AT_1 receptor. $Tyr^{35(TM1)}$ and the naphthyridin-2-one moiety of ZD7155 form hydrogen bonds. $Trp^{84(TM2)}$ of AT_1 receptor forms π-π interaction with the naphthyridin-2-one moiety of ZD7155. Additionally, residues $Ile^{31(TM1)}$, $Val^{108(TM3)}$, $Leu^{112(TM3)}$, and $Tyr^{292(TM7)}$ in AT_1 receptor ligand-binding pocket interact hydrophobically with ZD7155. Most of the other contacts for ZD7155 binding to AT_1 receptor, however, are mediated by residues, including $Tyr^{87(TM2)}$, $Thr^{88(TM2)}$, $Ser^{105(TM3)}$, $Ser^{109(TM3)}$, $Ala^{163(TM4)}$, $Phe^{182(ECL2)}$, $Pro^{285(TM7)}$, and $Ile^{288(TM7)}$ (Zhang et al., 2015).

Shape of the binding cavity is determined by the secondary structures and disulfide crosslinking patterns of the extracellular loop region, proline and non-proline kinks in 7TM helical bundle, and other local variations, resulting in deviations in the extracellular tips of TM helices. Specific structural variations create a remarkable variety of sizes, shapes, and electrostatic properties of the ligand-binding pockets in different GPCRs. The ZD7155 binding site in AT_1 receptor partially overlaps with known ligand binding sites in the chemokine and opioid receptors in which the cavity is larger, more open, and located closer to the extracellular surface, a general feature of GPCRs that recognize diffusible small-molecule ligands. Some of the residues that comprise the ligand-binding pockets are conserved among these structurally similar peptide GPCRs, including $Tyr^{(TM1)}$ and $Trp^{(TM2)}$, and the majority of the residues forming the ligand binding of these receptors are close to the extracellular boundaries of the helices. $Arg^{167(ECL2)}$ is one of the three critical residues that holds the antagonist close to the extracellular side and it is a unique residue of AT_1 receptor compared with other structurally similar peptide GPCRs.

Docking simulations of the clinically used antihypertensive ARBs show that they bind in similar orientations and interact with the three critical residues, $Arg^{167(ECL2)}$, $Trp^{84(TM2)}$, and $Tyr^{35(TM1)}$ in the AT_1 receptor ligand-binding pocket (Fig. 3). Details of the interactions with residues $Phe^{77(TMII)}$, $Tyr^{87(TMII)}$, $Ser^{105(TMIII)}$, $Val^{108(TMIII)}$, $Ser^{109(TMIII)}$, $Leu^{112(TMIII)}$, $Ala^{163(TMIV)}$, $Phe^{182(ECL2)}$, $Ile^{288(TMVII)}$, and $Tyr^{292(TMVII)}$, which shape the ligand-binding pocket of the AT^1 receptor, are different for different ARBs. For example, one of the common features among these ARBs is a short alkyl tail with 2–4 carbons extending into a narrow hydrophobic pocket formed by $Tyr^{35(TM1)}$, $Phe^{77(TM2)}$, $Val^{108(TM3)}$, $Ile^{288(TM7)}$, and $Tyr^{292(TM7)}$. Losartan is a surmountable antagonist with lower binding affinity to AT_1 receptor compared with the later developed ARBs (Takezako et al., 2004; Miura et al., 2011). Docking results suggest that $Arg^{167(ECL2)}$ forms a salt bridge with only the tetrazole moiety of losartan but lacks other polar interaction and its interaction with $Tyr^{35(TM1)}$; distances and angles for hydrogen bonding are suboptimal. The lower binding affinity and surmountable property of losartan for AT_1 receptor is likely due to this. In contrast, candesartan docking results indicate that it forms optimal interaction with the pocket. $Lys^{199(TM5)}$ may form an additional salt bridge with the tetrazole moiety and further stabilize candesartan binding. Crystal structure and docking analyses suggest that $Lys^{199(TM5)}$ provides conformational heterogeneity in the AT_1 receptor; the amino group of this residue may reach the acidic moieties of ARBs by forming salt bridges (as in the cases of candesartan and telmisartan) or through water-mediated interactions with other ARBs (Zhang et al., 2015). The structure of nonpeptide antagonists mimics the contact points of AngII side chains, and their binding sites overlap with the AngII binding pocket in AT_1 receptor as also supported by mutagenesis and crosslinking experiments (Yamano et al., 1992; Ji et al., 1994; Noda et al., 1995b; Vanderheyden et al., 2000a; Takezako et al., 2004).

Current literature is dominated by studies on losartan, candesartan, valsartan, irbesartan, olmesartan, and azilsartan that share a common biphenyl-tetrazole scaffold. Two ARBs in clinical practice differ, however. Telmisartan (also known as BIBR 277) structure is unusual, with two benzimidazole moeities attached (Fig. 4A). Eprosartan (also known as SK&F 108566), is the other ARB with the most differentiated structure. Telmisartan and eprosartan treatment benefits are not fully characterized, but potential benefits due to differences in their structural features are possible. These ARBs interact $Arg167^{ECL2}$, $Tyr35^{TM1}$, and $Trp84^{TM2}$ similar to other biphenyl-tetrazole ARBs, and mutants of these residues dramatically reduced their binding. However, the three-dimensional structure modeling studies predicted that telmisartan and

eprosartan bind, interacting with significantly different residues (Fig. 4B). Eprosartan extends interaction into the hydrophobic subpocket consisting of $Ile288^{TM7}$ and $Tyr292^{TM7}$. Alanine substitution of both $Ile288^{TM7}$ and $Tyr292^{TM7}$ specifically decreased eprosartan-binding affinities. Two consecutive benzimidazole moieties in telmisartan were predicted to make additional π-π contacts with $Tyr92^{ECL1}$ (Fig. 4B). Mutation of $Tyr92^{ECL1}$ to alanine, although mostly neutral for other ARBs tested, significantly lowered affinity for telmisartan. Furthermore, Ile288Ala mutation also has a discriminating effect on telmisartan binding. Structural analysis reveals a novel paradigm, molecular recognition through extended interaction with receptor subpockets. This may suggest possible difference in outcome from blockade of AT_1 receptor function using different ARBs (Zhang et al., 2015).

2. Inverse Agonism. Classic competitive antagonism model does not easily explain pharmacological behavior of most ARBs, therefore, more recent models to describe drug properties have been adopted. It is now generally accepted that AT_1 receptor has small but functionally significant constitutive activity (Unal and Karnik, 2014), and perhaps most ARBs do reduce constitutive activity of receptor in the absence of agonist, a phenomenon called inverse agonism. Inverse agonism of most ARBs is detected using constitutively active mutants of AT_1 receptor created by site-directed mutagenesis (Unal and Karnik, 2014). Inverse agonism has been shown for EXP3174, olmesartan, telmisartan, valsartan, and azilsartan (Noda et al., 1996; Miura et al., 2003a, 2006, 2013; Feng et al., 2005; Bhuiyan et al., 2009; Ojima et al., 2011). Whether losartan possesses inverse agonist properties remains controversial, because it was noted in one report (Bhuiyan et al., 2009) but not in further studies (Miura et al., 2003b; Feng et al., 2005). Clinical relevance of inverse agonism of ARBs is still debated. Kiya et al. (2010) studied nephroprotective properties of olmesartan and its analog lacking inverse agonism in Dahl salt-sensitive rats. Olmesartan lowered urinary protein excretion by ~25%, but its close structural analog that lacked inverse agonism did not show nephroprotective effect. Differential effect of inverse agonist ARBs on proteinuria remains to be systematically studied.

3. Insurmountable and Reversible Antagonism. Most clinically used ARBs exhibit an atypical competitive and reversible interaction with AT_1 receptor. The AngII concentration-response curves in the presence of ARBs shift toward higher concentrations, but with reduced maximal response. In experimental settings, a very high concentration of AngII cannot overcome the ARB inhibition fully. Most clinically used ARBs harbor this behavior (van Liefde and Vauquelin, 2009), but insurmountable antagonism of ARBs remains a rather descriptive term. Site-directed mutagenesis studies to identify receptor mechanisms have not provided a satisfactory answer (Vanderheyden et al., 2000b; Verheijen et al., 2003; Van Liefde and Vauquelin, 2009). Insurmountable antagonism of candesartan (Noda et al., 1993; Ojima et al., 1997), olmesartan, or EXP3174 was shown to increase with duration of preincubation with the receptor (Mizuno et al., 1995). Antagonism lasted for hours upon washout for many ARBs, including candesartan, EXP3174, olmesartan, telmisartan, and azilsartan. In insurmountable antagonism, the slow dissociation rates from the receptor may suggest conformational effects of ARB binding with clinically relevant functional consequences. The dissociation half-lives of the hAT_1 receptor has been measured, and the order of dissociation was found to be telmisartan, olmesartan, candesartan, valsartan, and losartan, respectively, with $t_{1/2}$ of 213, 166, 133, 70, and 67 minutes (Vanderheyden et al., 2000b; Verheijen et al., 2000; Kakuta et al., 2005)

4. Biased Agonism. The ability of a given receptor to selectively activate a signaling path compared with another is defined as "biased agonism" or "ligand-directed" signaling. Biased ligands, also known as functionally selective agonists, can involve a subset of a receptor's normal signaling repertoire by stabilizing different receptor conformational states than are stabilized by endogenous "unbiased" ligands (Kenakin, 2007; Violin and Lefkowitz, 2007; DeWire and Violin, 2011; Godin and Ferguson, 2012). Functional selectivity exhibited by several AngII analogs has forced redefinition of receptor agonism paradigm to allow discrete receptor activation states from ligands with different efficacy for individual responses (Kenakin, 2005; Galandrin et al., 2007; Urban et al., 2007; Hansen et al., 2008; Lyngso et al., 2009). AngII peptide modification led to the description of agonists, partial agonists, and antagonists (Miura et al., 1999; Miura and Karnik, 1999; Oliveira et al., 2007). Hormone AngII is a full agonist with pluridimensional efficacy for all signals activated by AT_1 receptor, whereas AngII analog such as $[Sar^1,Ile^4,Ile^8]$ AngII (SII-AngII) is a biased agonist that does not activate G protein signaling but allows receptor phosphorylation and subsequent β-arrestin–mediated signaling (Kenakin, 2003, 2005; Urban et al., 2007). This discovery has opened the possibility for novel designer ligands that may activate only some of AT_1 receptor signals. This development holds great promise for the treatment of diseases such as HF and hypertension (Holloway et al., 2002; Wei et al., 2003; Daniels et al., 2005; Aplin et al., 2007a,b; Hansen et al., 2008; Shukla et al., 2008; Sauliere et al., 2012).

Insight into the pluridimensional efficacy of AngII-analogs was initially described by Thomas et al. (2000) and Holloway et al. (2002). These authors studied the IP3 signaling, MAPK signaling, phosphorylation, and internalization of AT_1 receptor in response to AngII and several AngII analogs. Internalization of AT_1 receptor-EGFP was unaffected by substitution of Tyr-4

and Phe-8 in AngII that abolish IP3 signaling (Holloway et al., 2002). On the other hand, substitution of Asp-1 with alanine and substitutions of Phe^8 with alanine, isoleucine, diphenylalanine, and β-cyclohexylalanine significantly inhibited phosphorylation. The activation of MAPK was inhibited by Phe-8 substitutions, which did not equally inhibit IP3 production or receptor phosphorylation, indicating overlapping but distinct AngII-AT_1 receptor interactions. Yee et al. (2006) described three distinct activated states of AT_1 receptor based on the response of several AT_1 receptor mutants to AngII and SII-AngII. Three active states proposed include AngII-mediated G-protein signaling, AngII-mediated G-protein–independent ERK1/2 signaling, and II-AngII–mediated G-protein–independent ERK1/2 signaling (Yee et al., 2006), thus linking receptor ligand combinations to generation of observed functional selectivity. SII-AngII is extensively studied for engagement of GRKs, antiapoptotic signals, chemotaxis, cell growth, and proliferation (Hunton et al., 2005; Yee et al., 2006; Aplin et al., 2007a,b; DeWire et al., 2008). An analog such as TRV120027 is a 30-fold more potent β-arrestin–biased AT_1 receptor ligand that has been studied in vitro and in vivo in rats and dogs and is now in Phase II clinical studies for the treatment of acute HF. The preclinical data are reported to indicate that TRV120027 has the potential to support heart, vasculature, and kidney function in acute HF (Violin et al., 2010; Boerrigter et al., 2011). TRV120027 may block the effects of elevated AngII on the vasculature and kidney, while protecting or enhancing prosurvival and contractility signals in the heart.

As seen in the case of many GPCR antagonists, ARBs do not cause internalization of the AT_1 receptor and block agonist-induced internalization. Unbiased antagonism by losartan, telmisartan, and valsartan are reported in internalization and desensitization (Violin et al., 2010). However, candesartan, losartan, telmisartan, and valsartan allow substantial internalization of constitutively active AT_1 receptor mutants (Bhuiyan et al., 2010). This phenomenon may reflect hidden biased agonism by these ARBs, a phenomenon that needs to be rigorously evaluated. Instances of a compound acting as an antagonist for one response and an agonist for a different response mediated by the same receptor are known (Patel et al., 2012). These findings contrast with studies in which candesartan binding did not induce internalization of a GFP-AT_1 receptor in Chinese hamster ovary (CHO) cells (Le et al., 2005). However, these aspects are yet to be described for AT_1 receptor and its peptide as well as nonpeptide ligands.

C. Mouse Models

No major abnormalities of the cardiac and vascular system are reported in mice lacking the AT_{1a} receptor (AT_{1a} receptor-null). These mice develop normally but show a marked reduction of systolic blood pressure (Yang et al., 2010). In contrast, the AT_{1b} receptor-null mice are normal, confirming that AT_{1b} receptor has a negligible role normally. For instance, in the AT_{1a}R deleted mice, AT_{1b} receptor substitutes by mediating calcium signaling in vascular smooth muscle cells (VSMC) (Zhu et al., 1998). Animals with both AT_{1a} receptor and AT_{1b} receptor deletion have increased mortality, impaired growth, hypotension, and marked abnormalities in renal structures (Ito et al., 1995; Sugaya et al., 1995; Chen et al., 1997; Oliverio et al., 1998). There is a complete absence of pressor responses to AngII in the double knockout mice. Kidney functions are mostly affected in AT_{1a} receptor mice, with mild mesangial expansion and juxtaglomerular cell hypertrophy. However, circulating AngII levels are not elevated in these mice. Similarly, vascular tone is not altered through other systems. AngII can elicit an attenuated renal vasoconstriction in AT_{1a} receptor-null mice (Ruan et al., 1999). AT_{1a} receptor knockout causes polyuria and defective urine concentration in mice by reducing vasopressin signaling in the inner medulla (Li et al., 2009c). Structural abnormalities in the vascular system are observed in AT_{1a} receptor mice. Dysfunction in the renal vascular system and change of VSMC proliferation rate, altering the circular mechanical integrity of vessels, and increased synthesis of extracellular matrices are observed (Inokuchi et al., 2001). Ischemia-induced angiogenesis was also impaired, suggesting that AT_{1a} receptor-null condition affects early angiogenesis through inflammatory cell infiltration and angiogenic cytokine expression (Sasaki et al., 2002) and a delay in wound healing (Kurosaka et al., 2009). Reduced differentiation and hypertrophy of adipocytes in AT_{1a} receptor-null mice was also observed (Sasaki et al., 2002). Heart tissue appears to show decrease of risk in the AT_{1a} receptor-null mice, with reduced remodeling of left ventricular wall associated with improved survival after experimental myocardial infarction (Harada et al., 1999). In aortic regurgitation models of AT_{1a} receptor-null mice, long-term survival is improved by attenuating the progression of left ventricule dilatation, hypertrophy, and fibrosis (Nakanishi et al., 2007). Restoring the expression of AT_{1a} receptor in the C1 neurons of AT_{1a} receptor knockout mice reinstates the sympathoexcitation response to AngII in the rostral ventrolateral medulla (Chen et al., 2010).

Transgenic mice overexpressing AT_{1a} receptor in specific tissues and cells have been developed. Cardiac targeted AT_{1a} receptor overexpression generated a wide spectrum of effects, some of which appear to be strain dependent. Severe cardiac hypertrophy, failure, and death observed at fetal stage is associated with hyperplasia myocytes and heart block (Hein et al., 1997). In rats, similar transgenesis, however, appear to produce normal heart functions. However, cardiac hypertrophy and contractile response to AngII was augmented after volume and pressure overload in these rats (Hoffmann

et al., 2001). Pressure- or volume-overload causes more pronounced hypertrophy in transgenic rats than in normal rats. Cardiac targeted AT_1 receptor overexpression in C57BL/6 mice induces cardiac hypertrophy and remodeling with increased atrial natriuretic factor secretion and interstitial collagen deposition and premature HF. Systolic blood pressure and the heart rate was normal in the transgenic mice (Paradis et al., 2000). Impaired excitation-contraction coupling in heart before the development of cardiac hypertrophy is reported (Rivard et al., 2011). A decrease in myocardial microvessel density after experimental myocardial infarction was observed. Therefore benefit of ARB treatment of myocardial infarction may be due to a stimulatory effect on myocardial angiogenesis (de Boer et al., 2003). Overexpression of AT_1 receptor in the cardiac myocytes of angiotensinogen-knockout mice presented spontaneous systolic dysfunction, chamber dilatation, and severe interstitial fibrosis. Treatment with candesartan, an inverse agonist for the AT_1 receptor, prevented progressive cardiac remodeling in this model. This proof-of-principle study demonstrated that the basal constitutive activity of the AT_1 receptor contributes to the cardiac remodeling, in complete absence of AngII, when level of AT_1 receptor is increased in the heart (Yasuda et al., 2012).

Overexpression of AT_{1a} receptor restricted to brain enhanced cardiovascular responsiveness to intracerebroventricular injection of AngII without a change in baseline blood pressure. However, with intracerebroventricular injection of losartan to block the central AT_{1a} receptor reduced basal blood pressure, suggesting an enhanced contribution of central AT_{1a} receptor to the maintenance of baseline blood pressure (Lazartigues et al., 2002). Renovascular hypertension in these mice is buffered by increased nitric oxide production in the peripheral vasculature (Lazartigues et al., 2004). An enhanced salt appetite and altered water intake is observed upon brain-selective overexpression of AT_{1a} receptor (Lazartigues et al., 2008).

AT_1 receptor overexpression in transgenic rats directed to podocytes induces protein leakage and structural damage to podocytes, progressing to focal segmental glomerulosclerosis (Hoffmann et al., 2004). Mice overexpressing a constitutively active AT_1 receptor transgene in renal proximal tubule caused increased baseline blood pressure. Depletion of endogenous AT_{1a} receptor in the proximal tubule reduced blood pressure. However, there was no difference in the blood pressure response to a pressor dose of AngII in either experimental model, suggesting that the AT_{1a} receptor in the renal proximal tubule is a regulator of systemic blood pressure under baseline conditions (Li et al., 2011).

Transgenic overexpression of the constitutively active N111G mutant AT_1 receptor restricted to vascular endothelium significantly reduced the pressor response of carotid artery to acute infusion of AngII, resulting in hypotension and bradycardia (Ramchandran et al., 2006). Increased nitric oxide synthase expression in the endothelial cells seems to increase NO in blood and moderate response of smooth muscle cells to AngII. Gene knock-in expression of the constitutively active N111S mutant of AT_1 receptor with a C-terminal deletion produced long-lasting pressor response to infused AngII and a moderate and stable increase in blood pressure (Billet et al., 2007). These mice develop diastolic dysfunction without developing significant cardiac hypertrophy but show progressive renal and cardiac fibrosis. Overexpression of the constitutively active N111G mutant of AT_1 receptor in cardiac myocytes produced enhanced myocyte growth from the onset of adolescence associated with cardiac hypertrophy in the adult without progressing to pathologic remodeling or HF (Ainscough et al., 2009). However, AngIV peptide infusion induced adverse ventricular remodeling within 4 weeks characterized by increased interstitial fibrosis, dilatation of the left ventricle, and impaired cardiac function.

Transgenesis for inhibition of the RAS at a genetic level, which involves an antisense targeting AT_1 receptor, has been proposed as therapy for chronic control of blood pressure. A retrovirus-based delivery of AT_1 receptor antisense was reported to prevent hypertension in the spontaneously hypertensive rats (Reaves et al., 2000; Wang et al., 2000b) and protects normotensive rats from developing AngII-infused hypertension (Pachori et al., 2000). Intracerebroventricular injections of antisense oligonucleotides targeting AT_1 receptor in the brain decreased the blood pressure in chronic 2-kidney, 1-clip hypertensive rats (Kagiyama et al., 2001). Antisense inhibition of the AT_1 receptor in human pulmonary artery smooth muscle cells has powerful inhibitory effects on AngII-induced migration, proliferation of, and promotion of apoptosis (Tu et al., 2005). These studies suggest that antisense inhibition targeting to AT_1 receptor has therapeutic potential for the treatment of vascular diseases, including hypertension and hypertension-associated cardiac and vascular pathophysiology.

D. Genetic Polymorphism

The AGTR1 gene is highly polymorphic (Duncan et al., 2001; Mottl et al., 2008). In particular, nucleotide 1166 A/C transversion in the 3′ untranslated region of the gene (Bonnardeaux et al., 1994) has been associated with essential hypertension, increased aortic stiffness (Benetos et al., 1995), and myocardial infarction (Berge et al., 1997), with large interethnic, age, and sex differences in the frequencies. At least 50 single nucleotide polymorphisms (SNPs) have been described, among which nine of them are in the functional promoter region of the gene having the potential to influence AT_1 receptor gene expression (Erdmann et al., 1999). The significance of AT_1 receptor polymorphisms in

essential hypertension remains controversial (Griendling et al., 1996; Luft, 2004). Association of A1166C with increased sensitivity for AngII was reported in hypertensive patients on a high-salt diet (Spiering et al., 2000). A1166C polymorphism was associated with hypertension-related impairment of renal function (Buraczynska et al., 2002; Coll et al., 2003). A1166C was associated with enhanced vasoconstriction by AngII in isolated human arteries (van Geel et al., 2000). The A1166C polymorphism may increase the risk of coronary heart disease in patients with familial hypercholesterolemia (Wierzbicki et al., 2000). The association of A1166C polymorphism with losartan treatment in hemodynamic response measurement should be important for further research to understand the individual responses to a variety of AT_1 receptor blockers (ARBs) and develop personalized antihypertensive therapy (Baudin, 2002).

Naturally occurring amino acid variations in hAT_1 receptor are reported in genome databases (http://www.uniprot.org/uniprot/P30556). These include miss-sense variations, L48V, A163T, L222V, A244S, T282M, C289W, T336P, P341H. Of these miss-sense variations, A163T, T282M, and C289W may directly affect ligand binding, and L48V, L222V, and A244S may indirectly influence ligand binding or signaling by AT_1 receptor (see Fig. 5). Variant residues, T336P and P341H are located in the C-terminal tail that is not included in the crystalized AT_1 receptor. However, these residues are known to be phosphorylated, an event that is necessary for β-arrestin recruitment to AT_1 receptor and subsequent receptor trafficking to endosomes. The T282M variant is linked to renal tubular digenesis syndrome by an unknown mechanism (Gribouval et al., 2005) and the Thr163 variant lowered affinity for losartan (Arsenault et al., 2010b). The AT_1 receptor crystal structure indicated that 14% of $Ala163^{TM4}$ side-chain surface interacted with ARBs. $Cys289^{TM7}$ faces ARB binding pocket but does not interact with ARBs, and $Thr282^{TM7}$ is not in the ARB pocket. Mutagenesis studies have shown that residues located closely to the binding site reduce the affinity of ARBs and AngII but not $Thr282^{TM7}$ and $Cys289^{TM7}$, suggesting that variants may not directly alter ligand-receptor interactions (Zhang et al., 2015). However, the variant residues in humans are different from those evaluated in mutagenesis studies; therefore variant residue effects on ligand binding need to be determined through experiments. Phenotypic effects of other variant residues are difficult to predict, but may affect AngII and antihypertensive response in individuals carrying these variations.

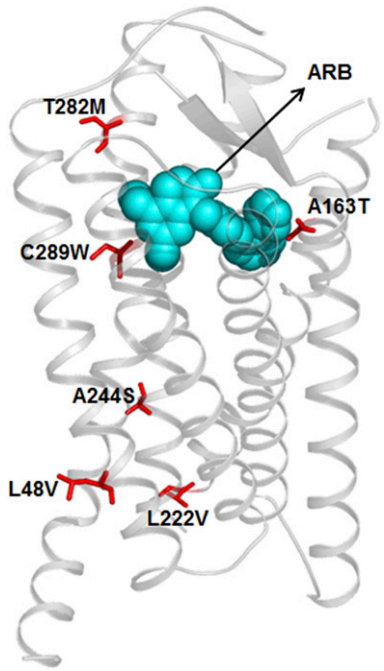

Fig. 5. Single residue variations of hAT_1 receptor in population. Location of reported variant residues are shown in an ARB bound three-dimensional model of the receptor.

E. Signaling

Over the past 15 years, the AT_1 receptor signaling has been studied in great detail (see Fig. 6) to understand mechanism of regulation of vasoconstriction, sodium reabsorption, cell proliferation, extracellular matrix formation, inflammation, and oxidative stress by RAS and how ARBs might intercept signaling during pathology (De Gasparo, 2002; Lefkowitz and Shenoy, 2005; Rajagopal et al., 2005; Hunyady and Catt, 2006; Mehta and Griendling, 2007; Oliveira et al., 2007; Oro et al., 2007; Violin and Lefkowitz, 2007; Aplin et al., 2009; Lyngso et al., 2009). AT_1 receptor is the lead example for establishing a novel cell signaling principle that a single GPCR ligand can activate multiple signaling pathways both dependent and independent of heterotrimeric G-proteins with differing efficacies (Kenakin, 2001; Lefkowitz and Shenoy, 2005; Violin and Lefkowitz, 2007; Patel et al., 2010).

The traditionally portrayed signaling mechanism of the AT_1 receptor is dependent on heterotrimeric G proteins (Hunyady and Catt, 2006). In addition to coupling with the heterotrimeric G-proteins, AngII activates both nonreceptor and receptor tyrosine kinases (Ishida et al., 1995; Sadoshima and Izumo, 1996; Bernstein et al., 1998; Eguchi et al., 1998; Sadoshima, 1998; Heeneman et al., 2000; Saito and Berk, 2001; Higuchi et al., 2007). These interactions orchestrate pleotropic signaling in cells that include enzymes, adapter proteins, transcription factors, and small GTP binding proteins and downstream kinases, accounting for a wide spectrum of responses to AngII (Marrero et al., 1995b; Venema et al., 1998b).

1. G-protein–Mediated Signaling. Multiple heterotrimeric G-proteins interact with the AT_1 receptor, including $G_{q/11}$, G_i, G_{12}, and G_{13}, leading to activation of downstream effectors including phospholipase C (PLC), phospholipase A, and phospholipase D (Shirai et al., 1995; Ushio-Fukai et al., 1999a; Higuchi et al., 2007).

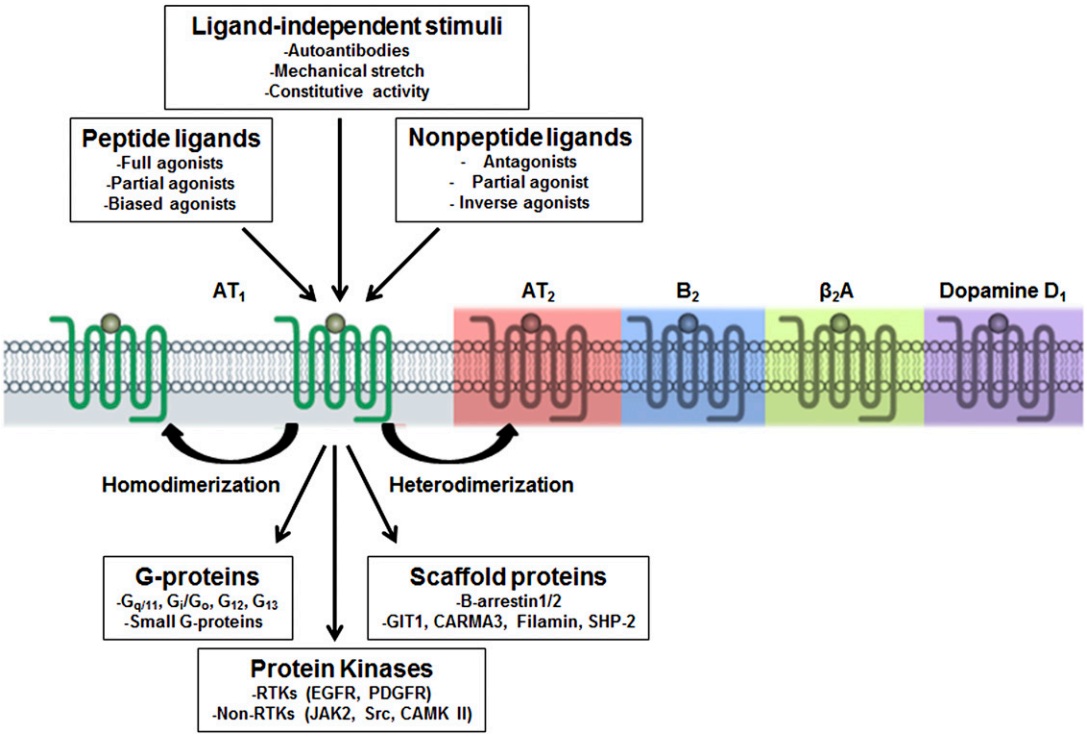

Fig. 6. Schematic representation of AT_1 receptor as a pluridimensional signal transducer. Discoveries made in the past 15 years suggest that AT_1 receptor as a transmembrane transducer capable of sensing multiple modes of stimuli and elicit diverse responses as shown.

The AT_1 receptor-$G_{q/11}$-phospholipase Cβ (PLCβ) coupling results in inositol triphosphates (IP3) and diacylglycerol (DAG) (Yusuf et al., 2000) signals. IP3 causes release of Ca^{2+} from the intracellular store. Intracellular Ca^{2+} cycling is the primary trigger for excitation-contraction of both cardiac and vascular myocytes. In addition, cytosolic Ca^{2+} does trigger intracellular signaling through calcineurin/nuclear factor of activated in T-cells or the calcium/calmodulin-dependent protein kinase II (CamK II) cascades, which are relevant to hypertrophy (Heineke and Molkentin, 2006). Histone deacetylase-5 has emerged as an important substrate of CamK II. A scaffold protein, GIT1, mediates AngII-induced VSMC gene transcription via CamK II-dependent phosphorylation and activation of HDAC5 (Pang et al., 2008). DAG activates PKC (Vallega et al., 1988), which also contributes to the vasoconstrictive and growth promoting effects of AngII. Phospholipase D–mediated phosphatidylcholine to choline and phosphatidic acid production is considered a second wave of signal by AngII activated AT_1 receptor. PA is rapidly converted to DAG, leading to sustained muscle contraction (Mehta and Griendling, 2007). AT_1 receptor causes activation of phospholipase A2 by phosphorylation and production of arachidonic acid and its metabolites. This signal is important in maintaining a balance between vasoconstriction and vasodilation in various vascular beds (Sarkis et al., 2004; Campbell et al., 1996) and NAD(P)H oxidation in VSMC (Griendling et al., 2000).

The $G_{\beta\gamma}$ subunits released upon AT_1 receptor activation have been shown to activate tyrosine kinases such as SHC, pp60$^{c\text{-}src}$, and JAK2 (Gutkind, 1998; Gschwind et al., 2001; Kranenburg and Moolenaar, 2001; Luchtefeld et al., 2001), leading to downstream phosphatidylinositol 3′-kinase γ ($PI3K_\gamma$) activation (Lopez-Ilasaca et al., 1997). AngII-induced activation of AT_1 receptor specifically augmented $G_{\beta 2}$ levels in the nucleus, where $G_{\beta 2}$ interacts with specific nucleosome core histones and specific chromatin bound transcription factors such as myocyte enhancer factor 2 and thereby regulates functional gene networks (Bhatnagar et al., 2013).

2. G-Protein–Independent β-Arrestin-Mediated Signaling. Multiple lines of observations led to the discovery that AT_1 receptor can directly recruit β-arrestin and mediate ERK1/2 signaling without G-protein activation (Holloway et al., 2002; Gaborik et al., 2003; Wei et al., 2003; Ahn et al., 2004a; Lee et al., 2008). The classic concept is that β-arrestin terminates G-protein signaling by blocking the receptor and stimulating receptor desensitization and endocytosis. However, β-arrestin–bound receptor can engage in G-protein–independent signaling, leading to distinct cellular responses (DeWire et al., 2007; Violin et al., 2013). This mode of signaling is recognized in a number of in vitro and in vivo settings; AT_1 receptor recruited β-arrestin1/2 engage a wide range of cellular responses.

The AT_1 receptor coupled β-arrestin mediates delayed ERK1/2 signaling confined to the cytoplasm for prolonged time ($t_{1/2} > 25$ min). In contrast, both nuclear

and cytoplasmic localization of activated ERK is G-protein–mediated (Gaborik et al., 2003; Tohgo et al., 2003; Ahn et al., 2004a). The AT_1 receptor-β-arrestin complex inhibits ERK-dependent transcription by binding to phospho-ERK and its retention in the cytosol (Tohgo et al., 2002). The cytoplasmic pool of ERK1/2 failed to phosphorylate the transcription factor Elk-1 and increase transcription of the immediate-early gene c-Fos (Tohgo et al., 2002; Aplin et al., 2007b). The β-arrestin2–dependent ERK activation may be responsible for distinct physiologic endpoints (Wei et al., 2004), such as increased survival of cardiac myocytes without entailing pathogenic myocyte hypertrophy. The beneficial effects of the β-arrestin–dependent pathway on enhancing cardiomyocyte survival was confirmed in a physiologic study in which a transgenic mouse with cardiac-specific overexpression of AT_1 receptor with second intracellular loop mutations that prevent G-protein coupling showed marked ventricular dilation and eccentric hypertrophy accompanied by diminished cardiomyocyte apoptosis in comparison with mice overexpressing a wild-type AT_1 receptor (Zhai et al., 2005).

Mechanistic details of activation of β-arrestin signaling by AT_1 receptor are emerging. AT_1 receptor can bind both β-arrestin1 and 2 (Oakley et al., 2000). Ahn et al. (2004b) demonstrated that physiologic levels of β arrestin1 might antagonize β-arrestin2–mediated ERK activation. Zimmerman et al. (2012) showed that AngII analogs selectively promoted β-arrestin–dependent effects in VMSCs. A mass spectrometry-based proteomics approach discovered that β-arrestin interacts with several proteins selectively after stimulation of the AT_1 receptor, indicating its potential for signaling (Xiao et al., 2007). In another study, activation by the β-arrestin biased ligand SII-AngII showed that unique phosphoproteins are activated (Christensen et al., 2010; Xiao et al., 2010). There are 34 differentially phosphorylated proteins, of which 16 were unique to SII-AngII and 8 were unique to AngII stimulation. Bioinformatics analysis of phosphorylated sites on these proteins identified downstream protein kinases activated by the AT_1 receptor-β-arrestin signaling (Kendall et al., 2011; Bogebo et al., 2014).

3. Reactive Oxygen Species Signaling. AngII is a potent mediator of oxidative stress and oxidant signaling (Ushio-Fukai et al., 1999b; Taniyama and Griendling, 2003; Yan et al., 2003a; Touyz, 2004). AngII activates membrane NAD(P)H oxidase mainly via AT_1 receptor and PKC to produce reactive oxygen species (ROS) like superoxide and hydrogen peroxide (H_2O_2) (Rajagopalan et al., 1996; Ushio-Fukai et al., 1996; Zafari et al., 1998; Griendling et al., 2000; Seshiah et al., 2002; Touyz et al., 2005). ROS are involved in many pleiotropic effects of AngII such as activation of signaling molecules (e.g., c-Src, EGFR, p38MAPK, Akt) and transcription factors [e.g., nuclear factor κB (NFκB), NF-κB, AP-1, Nrf2], which are involved in atherosclerosis pathology (Sen and Packer, 1996; Chen et al., 2006; Papaiahgari et al., 2006; Wu et al., 2005). In endothelial cells, a low amount of superoxide production by eNOS maintains a supply of NO, contributing to vasodilation and vascular health. In disease state, eNOS is uncoupled, leading to excessive superoxide that reacts with NO to form peroxynitrite, a toxic radical, which directly contributes to disease (Schena et al., 1999; Zhao et al., 2005a; Taguchi et al., 2011).

4. Nonreceptor Type Tyrosine Kinase Signaling. Nonreceptor tyrosine kinases associate with AT_1 receptor and target several intracellular proteins for phosphorylation. C-Src is a key player in AngII-mediated cellular effects. C-Src tyrosine kinase activity is also activated by ROS and it is involved in sustained calcium release (Sadoshima, 1998). Src is activated by AngII in an AT_1 receptor mutant lacking G-protein coupling ability, indicating that the carboxyl terminus of the AT_1 receptor is required for activation (Seta et al., 2002) because C-terminal truncation (310–359) abolished Src activation. VSMC growth is mediated by hyperactivation of c-Src-ERK1/2–dependent pathways by AT_1 receptor, leading to c-fos and AP-1 DNA-binding activity (Touyz et al., 2001b). AngII-stimulation of human monocyte migration is c-Src–dependent (Ishida et al., 1999) formation of focal adhesion complexes in the actin cytoskeleton (Polte et al., 1994). AT_1 receptor induces tyrosine phosphorylation of FAK, which then form a complex with Pyk2, p130Cas, paxillin, and talin, all of which interact to enable activation of cytoskeletal proteins facilitating adhesion of cells to extracellular matrix, and regulation of cell shape and movement (Leduc and Meloche, 1995; Sabe et al., 1997; Cary et al., 1998; Sayeski et al., 1998; Eguchi et al., 1999; Kintscher et al., 2001). Pyk2 is another kinase activated in response to AT_1 receptor and has been implicated in the regulation of ion channels, cellular adhesion, cell growth, and mitogenic and hypertrophic reactions (Sabri et al., 1998; Tang et al., 2000; Taniyama et al., 2003).

AT_1 receptor activates the JAK/STAT pathway via scaffolding by Src homology phosphatase-2 (SHP-2), a tyrosine phosphatase. The conserved YIPP motif in the AT_1 receptor interacts with SHP-2 and helps docking JAK2 and stimulates JAK2 phosphorylation at Tyr^{1007}/Tyr^{1008} (Marrero et al., 1995a; Doan et al., 2001; Frank et al., 2002; Godeny et al., 2007). Upon activation of JAK2 by AT_1 receptor, STAT proteins are activated in order to mediate gene transcription of early growth response genes, such as *c-fos* and *c-myc* (Berk and Corson, 1997; Ishida et al., 1999; Luttrell et al., 1999; Madamanchi et al., 2001). Another tyrosine phosphatase, SHP-1, causes JAK2 dephosphorylation and termination of the AngII-induced JAK/STAT signaling (Marrero et al., 1998). A calcium/PYK2 and PKC pathway also exists for JAK2 activation in VSMCs (Frank et al., 2002). An AT_1 receptor mutant with tyrosine 292, 302, 312, 319, and 339 mutated to phenylalanine uncoupled from

G-proteins was found to still activate tyrosine kinases and phospho-STAT1 signaling, indicating G-protein independence of these signals. JAK2 activation induces the expression of SOCS-3, which, in turn, blocks further activation of the pathway and consequently leads to desensitization of this signaling path (Torsoni et al., 2004).

5. Activation of Small G-protein Signaling. AngII also activates family of small G-proteins, such as Ras, Rho, and Rac through the AT_1 receptor (Ohtsu et al., 2006c), which regulate the MAPK cascades in cardiovascular remodeling induced by AngII. Ras is activated in both cardiac myocytes and VSMCs (Eguchi et al., 1996; Sadoshima and Izumo, 1996) by SHP-2 phosphorylation, which leads to Shc/Grb2 complex and recruitment of the guanine nucleotide exchange factor, son of sevenless. Subsequently son of sevenless activates the Ras/Raf/MEK/ERK1/2 pathway and c-fos transcriptional activity (Sugden and Clerk, 1997; Ohtsu et al., 2006c). PKC can also associate with Ras and activate ERK1/2 (Liao et al., 1996; Liao et al., 1997). Accumulating evidence suggests that the Rho/ROCK (Rho-kinase) pathway is crucial for AngII-induced remodeling of vasculature (Aoki et al., 1998). Rho pathways are involved in cell migration (Seko et al., 2003). Rac is implicated in activation of p21-activated kinase 1 by AngII in VSMCs, which subsequently mediates JNK activation and hypertrophy (Seko et al., 2003; Woolfolk et al., 2005; Ohtsu et al., 2006a). Rho pathway is involved in the Ca^{2+} sensitization of smooth muscle contraction, pre-myofibril formation, and expression of atrial natriuretic factor in cardiac myocytes. Rac is an important component of the reduced NADPH oxidase complex to produce ROS by AngII in VSMCs (Gregg et al., 2003). ERK1/2, JNK, and p38MAPK activated downstream of AT_1 receptor-NADPH pathway are implicated in VSMC differentiation, proliferation, and migration (Sugden and Clerk, 1997; Taniyama et al., 2004), as well as in fibrosis and target-organ damage (Ishida et al., 1998; Ishida et al., 1999). The phosphatase MAPK phosphatase-1 (MKP-1) serves as a negative feedback control, inactivating ERK1/2 (Bokemeyer et al., 1998).

6. Transactivation of Receptor Tyrosine Kinase Signaling. AT_1 receptor-induced transactivation of platelet derived growth factor receptor (PDGFR) modulates cell growth and migration (Heeneman et al., 2000; Suzuki and Eguchi, 2006) in VSMCs and mesangial cells (Linseman et al., 1995; Mondorf et al., 2000). This response is blocked by losartan and other ARBs. Upon acute AngII infusion, activation of PDGFR occurs in the vasculature of mice and rats. ACE-inhibitor infusion reduced aortic PDGFR phosphorylation and ERK activity (Kim et al., 2000), implicating PDGFR as downstream modulator of hypertensive vascular remodeling in vivo (Linseman et al., 1995; Heeneman et al., 2000; Mondorf et al., 2000; Gao et al., 2006).

AngII infusion also leads to activation of epidermal growth factor receptor (Zhang et al., 2009) in the vasculature (Kim et al., 2000). A major mechanism by which AngII influences growth-signaling pathways is through transactivation of EGFR by AT_1 receptor. AngII-induced renal deterioration involves EGFR transactivation mediated by ADAM17 (Lautrette et al., 2005) and second messengers such as Ca^{2+} and ROS (Diaz-Rodriguez et al., 2002; Mori et al., 2003; Seals and Courtneidge, 2003; Fischer et al., 2004; Tanaka et al., 2004; Mifune et al., 2005; Ohtsu et al., 2006a; Zhang et al., 2006). AT_1 receptor activation enhances the release of heparin-binding epidermal growth factor, which is dependent on A Disintegrin and Metalloproteinase (ADAM) family metalloproteinases (ADAM17) and Src (Andreev et al., 2001; Eguchi et al., 2001; Uchiyama-Tanaka et al., 2001; Schafer et al., 2004; Shah et al., 2004; Blobel, 2005; Mifune et al., 2005; Ohtsu et al., 2006a,b). Furthermore, dominant-negative ADAM17 mutant distinctly inhibited VSMC hypertrophy, which was stimulated by AngII (Ohtsu et al., 2006b). Heparin-binding epidermal growth factor activates EGFRs, allowing autophosphorylation on tyrosine (Prenzel et al., 1999). AngII-induced EGFR transactivation requires ROS and upstream kinases, such as c-Src, c-Abl, or Pyk2 (Dikic et al., 1996; Bokemeyer et al., 2000; Seshiah et al., 2002; Gratton et al., 2004) and leads to activation of the Ras/Raf/ERK pathway. AngII activation of Akt/PKB, p70S6K, and p38MAPK and induction of c-Fos leading to growth and migration of VSMCs, survival, and remodeling are mediated by EGFR (Che and Carmines, 2002; Seshiah et al., 2002; Suzuki et al., 2005).

A β-arrestin–dependent mechanism for transactivation of EGFR by AT_1 receptor has been reported (Kim et al., 2009). Calcium-independent AngII pathways also cause EGFR transactivation (Murasawa et al., 1998; Wang et al., 2000a) and the biased AngII analog [Sar^1, Ile^4,Ile^8]AngII activated G_q-independent EGFR signaling in human coronary artery smooth muscle cells (Miura et al., 2004). It was also reported that phosphorylation of tyrosine 319 and the YIPP motif of the AT_1 receptor is required for transactivation of EGFR (Seta and Sadoshima, 2003; Zhai et al., 2006). Cardiac-specific overexpression of an AT_1 receptor with a mutation in the YIPP motif (Tg-Y319F) did not cause cardiac hypertrophy in transgenic mice, and expression of fetal-type genes was significantly lower in these mice. Infusion of AngII failed to induce hypertrophy in Tg-Y319F mice, also significantly less apoptosis and fibrosis was reported (Zhai et al., 2006; Smith et al., 2011). EGFR activation is reported to be necessary for AngII-mediated hypertension and left ventricular hypertrophy (Ahmad et al., 2009).

7. Signaling through AT_1 Receptor Interacting Scaffold Proteins. AT_1 receptor signaling occurs through the recruitment of scaffolding regulatory proteins. The carboxyl-terminal cytoplasmic region of the AT_1 receptor

recruits different proteins to regulate different aspects of AT_1 receptor physiology (Mogi et al., 2009; Horiuchi et al., 2012). AT_1 receptor-associated protein (ATRAP1) is a transmembrane protein expressed in various tissues including the kidney, aorta, heart, lung, testis, and at a lower level in the lung, liver, spleen, and brain. It interacts specifically with the C-terminal tail and enhances AngII-induced internalization of AT_1 receptor (Daviet et al., 1999; Cui et al., 2000; Guo et al., 2003; Oshita et al., 2006; Azuma et al., 2007). ATRAP1 is a negative regulator of classic G-protein signaling by AT_1 receptor (Lopez-Ilasaca et al., 2003; Tamura et al., 2007) and VSMC growth (Cui et al., 2000) and cardiomyocyte hypertrophy (Tanaka et al., 2005). ATRAP1 prevents VSMC senescence (Wislez et al., 1998; Guo et al., 2005). ATRAP1 transgenic mice exhibited decrease in cardiac hypertrophy, neointima formation, inflammatory response, and NADPH oxidase activity in the injured artery (Oshita et al., 2006; Wakui and Tamura, 2012). In contrast, ATRAP1-deficient (ATRAP1−/−) mice showed increased mean systolic blood pressure and plasma volume, which was associated with increased surface expression of AT_1 receptors in the renal cortex and increased proximal tubular function (Oppermann et al., 2010). Kidney-specific ATRAP1 transgenic mice exhibit hypertension and renal hypertrophy and failure (Oppermann et al., 2010), suggesting that renal ATRAP1 plays an important role in regulating intrarenal RAS. Mechanistic aspects of AT_1 receptor interaction with ATRAP1 and transfer of signal are not clear at this time (Cook et al., 2008).

8. Mechanical Stretch. AT_1 receptors were identified as mechanosensors in the myocardium by Issei Komuro's team by demonstrating agonist-independent activation of AT_1 receptor when stretch-stress is applied (Hunyady and Turu, 2004; Yasuda et al., 2008a; Mederos y Schnitzler et al., 2011). Mechanical stretch activated ERKs in the cardiomyocytes prepared from both neonatal and adult angiotensinogen-deficient mice (Zou et al., 2004), which could be inhibited by inverse agonist ARBs, such as candesartan. Mechanical stretch induces Janus kinase 2 and translocation of G-proteins into the cytosol (Zou et al., 2004; Yasuda et al., 2008a). The conformational changes in mechanically activated AT_1 receptor have been mapped (Shyu et al., 2001; Karnik et al., 2003; Yasuda et al., 2008b).

Sadoshima et al. (1993) initially reported that mechanical stretch causes secretion of AngII from cytoplasmic storage granules in cultured cardiac myocytes and that stretch-induced hypertrophic responses are completely dependent on the secreted AngII. However, several studies later showed that AngII partly mediates mechanical stress-induced hypertrophic responses (Yamazaki et al., 1995; Kijima et al., 1996). Therefore, mechanical stretch seems to directly activate unique intracellular signaling molecules. Yasuda et al. (2008b) showed that cell stretch leads to activation of the AT_1 receptor, which could be suppressed by candesartan, an inverse agonist. Thus, mechanical stress can directly change the conformation of the AT_1 receptor to increase the receptor's basal activity. This was followed by Zou et al. (2004), demonstrating that the AT_1 receptor can be activated by mechanical stress inducing cardiac hypertrophy in vivo in an AGT-null background.

Stretch-induced activation of the AT_1 receptor protects cells against induced apoptosis involving PKB/Akt signaling (Kippenberger et al., 2005). Mechanical stretch potentiates AngII-induced VSMCs proliferation in spontaneously hypertensive rat through an AT_1 receptor/EGFR/ERK-dependent pathway. These findings may provide new insights into growth-promoting mechanisms in vasculature in a hypertensive state (Liu et al., 2010). Mechanical stretch triggered an AT_1 receptor-dependent conformational change in β-arrestin similar to that induced by a β-arrestin–biased ligand (Rakesh et al., 2010). These findings were unique to the AT_1 receptor (and not seen with the β1 adrenergic receptors) and suggest that AT_1 receptor is able to sense membrane stretch and transmit the activated receptor signal to β-arrestin.

9. Signaling through Heterodimerization. The current view is that homo- and heterodimer formation of GPCRs could be important for some of the receptor functions (Lyngso et al., 2009). For example, the AT_1 receptor dimerizes with the bradykinin B2 receptor (Fig. 6), which enhances AngII signaling (AbdAlla et al., 2000, 2001b, 2005), contributing to AngII hypersensitivity in women with preeclampsia (AbdAlla et al., 2000, 2001b). The arrestin-biased ligand [Sar^1,Ile^4,Ile^8]AngII negatively regulates AT_1 receptor-B2R heterodimers by promoting sequestration of AT_1 receptor-B2R heterodimers (Wilson et al., 2013). Heterodimerization of AT_1 receptor with the MAS receptor and AT_2 receptor decreases AT_1 receptor-specific signaling (AbdAlla et al., 2001a; Kostenis et al., 2005; Canals et al., 2006; Santos et al., 2007). The AT_1 receptor can also form complexes with the β2 adrenergic receptors, and it is possible to effectively block dual receptor signaling using only a single receptor antagonist (Barki-Harrington et al., 2003). The AT_1 receptor also coimmunoprecipitates with the epidermal growth factor receptor (EGFR); dopamine D1, D3, and D5; and the endothelin B receptors (Zeng et al., 2003a,b, 2005a,b, 2006; Olivares-Reyes et al., 2005).

Evidence that AT_1 receptors can form dimers is supported by BRET analysis, suggesting homo- or oligomeric complexes in living cells that are unaffected by both agonists and antagonists (Hansen et al., 2004). Coexpression of signaling-deficient mutants results in functional receptors. AT_1 receptor wild-type G-protein coupling was diminished when coexpressed with defective mutant receptors, indicating "cross-inhibitory" association (Karip et al., 2007). Covalently crosslinked homodimer formation for AT_1 receptor is reported in isolated monocytes (AbdAlla et al., 2004) induced by

factor XIIIA transglutaminase involving Gln^{315} in the carboxyl-terminal tail of the AT_1 receptor. Hypertensive patients have increased homodimer levels (AbdAlla et al., 2004; Ogawa and Glass, 2004). Aldosterone produces a nongenomic endothelium-independent vasoconstrictor effect by enhancing intracellular transglutaminase activity and presumably inducing AT_1 receptor dimer formation in mesenteric arterioles perhaps due to transglutaminase-induced AT_1 receptor dimer formation (Yamada et al., 2008).

10. AngiotensinII Type 1 Receptor Signaling by Phosphorylation, Desensitization, and Internalization. G-protein signaling by activated AT_1 receptor is accompanied by rapid phosphorylation and internalization of AT_1 receptor (Thomas, 1999; Hunyady et al., 2000; Guo et al., 2001; Thomas and Qian, 2003). Defects in desensitization are implicated in vascular diseases; for example, hypertensive rats overexpress GRK 5, altering AngII responsiveness (Ishizaka et al., 1997). AngII-induced desensitization of AT_1 receptor is dependent on carboxyl-terminal residues 329–347 (Conchon et al., 1998). This region is Ser and Thr rich, and phosphorylation of these residues plays a key role in the desensitization of AT_1 receptor responses. AngII-induced phosphorylation of the AT_1 receptor is mediated by both PKC and G-protein–coupled receptor kinases (GRK), mainly GRK2 and GRK5 (Oppermann et al., 1996a,b; Smith et al., 1998a; Qian et al., 1999). Mutation of the key serine and threonine residues in 332–338 region significantly inhibits AT_1 receptor internalization (Hunyady et al., 1994; Thomas et al., 1995, 1998; Smith et al., 1998b; Qian et al., 2001). Mutating hydrophobic residues in helix VIII in C terminus also inhibits AT_1 receptor internalization (Thomas et al., 1995). A diacidic motif of Asp^{236}-Asp^{237} in the ICL3 of the AT_1 receptor is required for optimal AngII-induced phosphorylation of AT_1 receptor by GRKs and internalization (Olivares-Reyes et al., 2001). Complete deletion of the cytoplasmic tail inhibits internalization of AT_1 receptor (Chaki et al., 1994; Hunyady et al., 1994; Balmforth et al., 1995), and an STL motif (Ser^{335}-Thr^{336}-Leu^{337}) in this region plays a critical role but also requires residues Leu^{316} and Tyr^{319} (Hunyady et al., 1994; Thomas et al., 1995).

Internalization of the AT_1 receptor follows both β-arrestin–dependent and –independent mechanisms (Lefkowitz, 1998; Somsel Rodman and Wandinger-Ness, 2000; Kim et al., 2005a). Alanine substitution for Thr^{332}, Ser^{335}, Thr^{336}, and Ser^{338} preclude agonist-induced β-arrestin recruitment by AT_1 receptor and attenuated internalization (Luttrell et al., 2001; Qian et al., 2001; Kule et al., 2004). Dramatically reduced AT_1 receptor internalization was observed in mouse embryonic fibroblasts lacking both β-arrestin 1 and β-arrestin 2 (Kohout et al., 2001). β-Arrestins target AT_1 receptor to clathrin-coated pits by interacting with clathrin and the clathrin adapter 2 (AP2), which directly interact with the AT_1 receptor carboxyl terminus (Fessart et al., 2005). These interactions are regulated by c-Src and the ADP-ribosylation factor 6 (ARF6), a small GTP-binding protein (Fessart et al., 2005; Poupart et al., 2007; Zimmerman et al., 2009).

After endocytosis, the receptor induces specific β-arrestin–mediated cell signaling pathways, distinct from G-protein signaling (Kim et al., 2005a). Shah et al. (2002) showed that β-arrestin–mediated ERK activation is regulated by transactivation of the EGFR and activation of GRK 5 and GRK6 (Kim et al., 2005a) but not GRK 2 and 3. The selective receptor phosphorylation on different sites by the various GRK isoforms may have important implications. For example, GRKs 5 and 6 seem to mediate effects associated with physiologic consequences, which are different from GRK 2–mediated effects (Kim et al., 2005a). Inhibition of GRK 5 or 6 attenuates β-arrestin–mediated ERK activation, whereas it is not affected by knockdown of GRK 2 or 3 (Kim et al., 2005a). An internalization-deficient mutant of the AT_1 receptor with truncated carboxyl terminus can also produce these responses, implying that internalization per se is not necessary for β-arrestin–mediated signaling (Turner et al., 2001).

AT_1 receptors are internalized within 10 minutes, and 25% of internalized receptors are recycled back to plasma membrane and the remainders are degraded in lysosomes (Gunther et al., 1980; Griendling et al., 1987). The Rab family of proteins, specifically Rab 1, is associated with transport of AT_1 receptor from endoplasmic reticulum to Golgi to cell surface (Wu et al., 2003). AT_1 receptor traffic to early endosomes is dependent on Rab 5 interaction with the carboxyl terminus of the receptor (Daviet et al., 1999; Somsel Rodman and Wandinger-Ness, 2000; Seachrist et al., 2002). Rab 7-positive late endosomes promote AngII dissociation. Rapid receptor recycling back to the plasma membrane takes place in Rab 11-positive vesicles by a rapid PI3K-dependent pathway (Garcia-Caballero et al., 2001; Hunyady et al., 2002; Seachrist et al., 2002; Dale et al., 2004).

In addition to clathrin-dependent pathway, AT_1 receptor can be internalized via specialized microdomains called caveolae, associated with caveolin (Ishizaka et al., 1998). In VSMCs, AngII regulates the expression, biosynthesis, and phosphorylation of caveolins and promotes the translocation of AT_1 receptor to caveolin-enriched membrane fractions (Ishizaka et al., 1998).

F. Expression and Regulation

AT_1 receptors in all organs are sensitively regulated by a number of physiologic and pathophysiological factors (Kaschina and Unger, 2003; Elton and Martin, 2007; Higuchi et al., 2007). AngII, interferon, growth factors, estrogens, statins, nitric oxide, thyroid hormone, retinoic acid, and peroxisome proliferator-activated receptor can suppress transcription of the rat AT_{1a} receptor gene

in cultured rat VSMCs. In contrast, glucocorticoids, insulin-like growth factor, interleukin (IL)-6, erythropoietin, and progesterone induced an upregulation of expression in VSMCs. AngII accelerates AT_1 receptor mRNA decay in VSMCs, which is governed by interaction of phosphorylated calreticulin with the 3′ untranslated segment 2175–2195 of the AT_1 receptor mRNA (Nickenig et al., 2002). Regulation of rat AT_{1a} receptor promoter by cyclic AMP (Chen et al., 2002), free radicals (Nickenig et al., 2000; Chen et al., 2002), tumor necrosis factor-alpha (TNFα), interleukin-1 beta (Cowling et al., 2002), and purinergic P2Y(Dorn and Force, 2005) receptor in cardiomyocytes (Nishida et al., 2011) are observed.

Human and rodent genes for the AT_1 receptor are thought to be regulated by different mechanisms, because the promoter regions are divergent. Expression of the hAT_1 receptor is regulated predominantly by Sp1 and Sp3. MEF-2 and Sp1 regulate basal expression of the rat AT_{1a} receptor gene. PPAR suppresses rat AT_{1a} receptor gene the transcription by inhibiting Sp1 binding. Thus PPAR ligands may inhibit AngII-induced cell growth and hypertrophy in VSMCs by inhibiting hAT_1 receptor expression. In contrast, PPAR and CBP enhanced activity of Sp1 increase transcription of rat AT_{1a} receptor gene. Oxidized low-density lipoprotein (LDL) upregulates AT_1 receptor expression in cultured human coronary artery endothelial cells (Li et al., 2000).

G. Pathophysiological Aspects of AngII Type 1 Receptor Activation

AngII signaling through the AT_1 receptor promotes pathogenic processes such as ROS production, inflammation, altered vasoreactivity, growth, migration, platelet activation, and fibrosis. Ultimately these cause diseases such as hypertension, atherosclerosis, thrombosis, chronic kidney disease, and insulin resistance, with the final development of cardiovascular disease. Improved clinical outcomes after treatment with ARBs proves the causative role of AT_1 receptor in the pathogenesis of these diseases (Garg and Yusuf, 1995; Yusuf et al., 2000; Igarashi et al., 2001).

1. Cardiovascular Remodeling and Hypertrophy. In vitro and in vivo experiments have shown growth promoting actions of AngII, causing cardiac and vascular hypertrophy, cell differentiation, and apoptosis (Pfeffer and Braunwald, 1990; Lombardi et al., 1999; Lips et al., 2003; Dorn and Force, 2005). In general, ARBs effectively prevent cardiac, vascular, and renal hypertrophy (Kim et al., 1995, 1998). Involvement of ERK1/2, PI3K, and CDK2 inhibition pathways, leading to G1-phase arrest, causes myocyte hypertrophy (Braun-Dullaeus et al., 1999). Increase in protein synthesis involves activation of translation elongation factor-2 in cardiac myocytes via dephosphorylation by PP2A by a process that involves both PI3K and MAPK (Everett et al., 2001). Increased protein synthesis through AT_1 receptors in human cardiac fibroblasts did not induce hypertrophy of cardiac fibroblast (Hou et al., 2000). In the vasculature, DNA synthesis is enhanced upon AngII infusion through the activation of cyclin D1 and cdk4 and reduction in the expression of cell cycle kinase inhibitors p21 and p27 (Diep et al., 2001; Guillemot et al., 2000, 2001). Thus, in the myocardium, regulation of growth effects by AT_1 receptor in myocytes and fibroblasts differs. Cardiac hypertrophy includes cardiac myocyte enlargement and proliferation of cardiac fibroblasts. Now it is generally believed that both hypertrophic response of myocytes and proliferative response of fibroblast may depend on other modifying factors such as production of ROS and secretion of various types of factors.

For instance, in pressure overload due to hypertension and myocardial infarction, cardiac remodeling process includes cardiomyocyte hypertrophy, extracellular matrix synthesis, fibrosis, and loss of compliance, leading to fatal outcomes. TGF-β1 expression is increased in myocytes and fibroblasts of heart, which transdifferentiate into a myofibroblast phenotype, resulting in myocardial remodeling (Campbell and Katwa, 1997). The AT_1 receptor directly increases TGF-β1 expression (Kupfahl et al., 2000; Schultz et al., 2002), translocation of Smad proteins Smad 2 and 4 into the nucleus, resulting in expression of fibrotic marker proteins, collagen, fibronectin, and connective tissue growth factor (CTGF) (Hao et al., 2000; Rodriguez-Vita et al., 2005; Lim and Zhu, 2006; Zhang et al., 2009). CTGF is a profibrotic factor that stimulates both AngII- and TGF-β-1–mediated fibrosis and apoptosis (Abreu et al., 2002; Perbal, 2004; Cabello-Verrugio et al., 2011). CTGF is involved in myocardial remodeling mediated via AT_1 receptors during transition to HF (Iwanciw et al., 2003; Ahmed et al., 2004). AngII-induced CTGF production is also shown in the aorta of AngII-infused rats (Ruperez et al., 2003). AngII-stimulated collagen synthesis in aortic adventitial fibroblasts, which is actively involved in vascular remodeling, is mediated by CTGF (Che et al., 2008). Gene expression analysis detected high CTGF mRNA expression in coronary artery biopsies from ischemic injury and coronary artery disease. Proteins involved in extracellular matrix remodeling, such as thrombospondin 4, collagen type 1 and 2, and fibronectin, and the inflammatory cytokines, such as IL-8, IL-6, vascular cell adhesion molecule-1, and monocyte chemoattractant protein-1 (MCP-1), are believed to couple cardiac remodeling with chronic angiotensin receptor stimulation (Gabrielsen et al., 2007). IL-6 secretion by cardiac myocytes is regulated by AngII. The effects of IL-6 on cardiomyocyte hypertrophy and fibroblast proliferation is inhibited by the AT_1 receptor antagonist losartan, suggesting that IL-6 contribution to cardiomyocyte hypertrophy is mediated by the AT_1 receptor (Fredj et al., 2005). AngII-induced activation of the JAK/STAT pathway is involved in tissue remodeling

after vascular injury and myocardial ischemia in rats (Seki et al., 2000; Omura et al., 2001). The AT_1 receptor activates STAT1 and GATA4 transcription factors in the development of myocyte hypertrophy (Wang et al., 2005). Chronic activation of the AT_1 receptor in myocytes induces transcription of the Stat3 gene by pSTAT3 and overproduction of STAT3 protein, leading to nuclear accumulation of STAT3 without tyrosine phosphorylation, which alters the transcriptional program of cardiac hypertrophy (Yue et al., 2010).

VSMC hypertrophy induced by AngII involves PKC delta activation through Src-dependent Tyr phosphorylation, leading to Akt activation and signifying a novel molecular mechanism for enhancement of cardiovascular diseases induced by AngII (Nakashima et al., 2008). Antiapoptotic effects of AngII in cardiomyocytes and VSMCs are regulated by a mechanism involving PI3-kinase/Akt activation, subsequent upregulation of survivin, and suppression of caspase-3 activity (Ohashi et al., 2004). Inhibitors of Akt and a dominant-negative mutant of Akt selectively block AngII-induced proliferation of CHO-AT_{1a} cells (Dugourd et al., 2003). AngII activated reactive oxygen species acting through Src/caveolin-EGFR signaling pathway induces epithelial-to-mesenchymal transition in renal epithelial cells. This may be a novel molecular mechanism involved in progressive renal injury caused by chronic exposure to AngII (Chen et al., 2012). Adenoviral-directed expression of the AT_1 receptor has defined the EGFR transactivation pathway for cardiac hypertrophy via PI3K/Akt signaling (Ebert et al., 1995; Thomas et al., 2002).

2. Vascular Inflammation and Atherosclerosis. The role of AngII in atherosclerosis has been well established. Atherosclerotic risk factors such as hypercholesterolemia and hypertension also increase production of angiotensinogen (Daugherty et al., 2004). Inhibition of AT_1 receptor s by losartan prevents lipid peroxidation, decreasing atherosclerotic lesion formation n apolipoprotein E–deficient mice (Keidar et al., 1997). Conversely, AngII infusion increases aortic atherosclerosis and aneurysm formation, independent of blood pressure (Daugherty et al., 2000; Weiss et al., 2001; AbdAlla et al., 2001a). Male apoE/AT_{1a} receptor double knockout mice have reduced atherosclerosis (Wassmann et al., 2004a). This relationship between AT_1 receptor and atherosclerosis is conserved in hypercholesterolemic rabbits (Yang et al., 1998). Stimulation of inflammatory mediators including IL-1β, IL-6, and TNFα through NFκB activation (Sanz-Rosa et al., 2005) and RAS activation appears to be the pathogenic mechanism in the atherosclerotic process. AngII and/or IL-6 infusion induces oxidative stress and endothelial dysfunction in mice and these effects are completely abolished in AT_1 receptor knockout mice (Schieffer et al., 2000; Ruiz-Ortega et al., 2001b; Wolf et al., 2002; Skurk et al., 2004; Wassmann et al., 2004b). In VSMCs, the activation of NAD(P)H oxidase by the AT_1 receptor produces IL-6 (Marui et al., 1993; Chen et al., 1998; Kranzhofer et al., 1999) and AT_1 receptor blockers decreases TNFα, IL-6, and soluble adhesion molecules (Tsutamoto et al., 2000). Secretion of inflammatory factors, such as P-selectin and MCP-1 that are involved in the vascular inflammation and atherogenesis is AT_1 receptor-dependent, and ARBs attenuate P-selectin and MCP-1 expression with concurrent reduction in intimal proliferation in mice (Chen et al., 2001). In hepatocytes, CARMA3/Bcl10/MALT1-dependent NFκB activation mediates AngII-responsive inflammatory signaling promoting pathologic liver fibrosis (McAllister-Lucas et al., 2007). CARMA1 and 3 are expressed in VSMC and endothelial cells, suggesting the potential for pathway similar to hepatocytes playing a role in vascular inflammation and atherosclerosis. The AT_1 receptor stimulates myofibroblasts to proliferate through activation of the NFκB transcription factor via a signaling pathway composed of CARMA3, Bcl10, and MALT1 (McAllister-Lucas et al., 2007).

Under chronic liver injury, AngII promotes pathologic liver fibrosis by stimulating hepatocytes and hepatic stellate cells to synthesize extracellular matrix proteins and secrete secondary cytokines. In rats, experimental models of liver fibrosis induced by bile duct ligation or carbon tetrachloride or choline deficiency, administration of irbesartan, olmesartan, telmisartan, candesartan, or losartan inhibited expression of collagens and TGFβ in stellate cells and reduced established liver fibrosis (Kurikawa et al., 2003; Ueki et al., 2006; Hirose et al., 2007; Yoshiji et al., 2009; Moreno et al., 2010; Kato et al., 2012). In clinical practice, however, the usefulness of treating liver fibrosis with ARBs remains contradictory (Schneider et al., 1999; Gonzalez-Abraldes et al., 2001; Lee, 2014). A handful of randomized controlled trials suggest that ARB treatment is a potentially useful therapeutic approach in patients with nonalcoholic fatty liver disease (Paschos and Tziomalos, 2012). The nonalcoholic fatty liver disease patients benefited from telmisartan and olmesartan treatment (Enjoji et al., 2008; Colmenero et al., 2009). In two independent pilot studies, administration of candesartan or losartan was shown to have an antifibrotic effect on patients with chronic hepatitis C (Sookoian et al., 2005; Ueki et al., 2009).

3. Endothelial Dysfunction. Endothelial dysfunction refers to impairment of endothelium-dependent vasodilation associated with progressive changes in cell adhesion and barrier function. Inactivation of NO by AT_1 receptor–induced ROS is at the center of endothelial dysfunction in hypertension, atherosclerosis, and cardiovascular diseases. In endothelial cells, eNOS produces NO and a low amount of superoxide to maintain vasodilation and a healthy vasculature. In disease state, redox-uncoupled eNOS is responsible for disproportionate production of superoxide relative to NO. The excessive superoxide reacts with NO to form peroxynitrite, a toxic

radical, which directly contributes to disease state. Endothelial cells in a disease state express adhesion molecules, which increase adhesion of circulating inflammatory blood cells into the vessel wall, leading to further increase in ROS production (Rajagopalan et al., 1996; Schena et al., 1999; Zhao et al., 2005a). ROS increases local RAS activation in the vasculature, enhancing AngII production in ECs, VSMCs, and fibroblasts and creates a mutual reinforcement loop between local RAS and ROS. The AT_1 receptor induces LDL receptor expression in ECs (Gryglewski et al., 1986; Rubanyi and Vanhoutte, 1986; Li and Mehta, 2000) and enhances the modification of LDL and the expression of its lectin-like receptor (LOX-1), which are critical events in atherosclerotic lesion formation (Chen et al., 2000a; Figueroa and Vijayagopal, 2002). Overall endothelial dysfunction is characterized by blunted endothelium-dependent vasodilation associated with enhanced contraction in hypertensive diseases (Dasgupta and Zhang, 2011).

4. Oxidative Stress. AT_1 receptor–regulated oxidative stress plays a major part in the initiation and progression of hyperlipidemia, diabetes mellitus, hypertension, ischemic heart disease, and chronic HF (Marui et al., 1993; Taniyama and Griendling, 2003). ROS activates nuclear factor κB (NFκB) and stimulates degradation of its cytoplasmic inhibitor, IκB, an essential step in the proinflammatory process (Pueyo et al., 2000). Blockade of NFκB ameliorates myocardial hypertrophy in response to infusion of AngII, and the effect of AngII was attenuated in mice with targeted disruption of the p50 subunit of NFκB (Kawano et al., 2005). NFκB gene expression results in increased levels of vascular cell adhesion molecule-1 and probably other genes involved in the early stages of atherosclerosis (Pueyo et al., 2000). AngII/AT_1 receptor/ NFκB pathway may be involved in brain ischemia by stimulating intercellular adhesion molecule-1 expression in brain microvascular ECs (Liu et al., 2006). Thus, AT_1 receptor-induced ROS production can change structure-function properties of the vasculature that is the central aspect of vascular pathology in hypertension and diabetes. Treatment with ARBs stimulates NO release in platelets and ECs indicating arterial antithrombotic effects of ARBs (Kalinowski et al., 2002).

5. Extracellular Matrix Deposition. AngII is a risk factor implicated in cardiac remodeling, and ARB treatment is protective (Nagata et al., 2002). AT_1 receptor signaling is increasingly recognized for its profibrotic effects in other tissues as well. Mechanism of fibrosis may differ in different tissues. In the heart, the AT_1 receptor upregulates TGF-β1, laminin, and fibronectin expression and contributes to increased cardiac fibroblast attachment to collagens I and III and increased focal adhesion kinase activity. Synthesis of the extracellular matrix proteins upon AT_1 receptor activation (Kato et al., 1991; Mifune et al., 2000) involves transactivation of EGFR-MAPK–dependent pathways (Ju and Dixon, 1996; Touyz et al., 2001a). Abnormal accumulation of proteoglycans is known in atherosclerotic lesions (Evanko et al., 1998; Iozzo, 1998), and treatment with ARBs induces proteoglycan changes that favor healthier cell adhesion, migration, and differentiation (Iozzo, 1998; Moriguchi et al., 1999; Sasamura et al., 2001). In aortic VSMCs and ECs as well as in cardiac cells, the AT_1 receptor–EGFR transactivation pathway also regulates fibronectin synthesis, production of matrix metalloproteinases and breakdown of collagen IV, expression of plasminogen activator inhibitor-1 (Feener et al., 1995; Chen et al., 2000b; Kawano et al., 2000; Nakamura et al., 2000). Regulation of PAI-1 by the AT_1 receptor seems to be important in many contexts; for instance, in human adipocytes, impairment of the fibrinolysis has been implicated in obesity (Skurk et al., 2001) and in reduced trophoblast invasion (Xia et al., 2002; Abbasi et al., 2005). AT_1 receptor blockade effectively reduces AngII-stimulated PAI-1 secretion (Sironi et al., 2001). In skin wound healing, AT_1 receptor–stimulated keratinocyte and fibroblast migration mediated by EGFR transactivation (Yahata et al., 2006) causes an increase in TGF-β1 and integrin protein levels (Thibault et al., 2001). This wound-healing pathway is attenuated in AT_1 receptor-knockout mice. Thus, the AT_1 receptor regulates formation of ECM components and turnover of matrix. The mechanisms and pathways that integrate ECM formation and turnover in relation to AngII signaling are still being discovered.

6. Insulin Resistance. Patients with an imbalance in RAS homeostasis exhibit decreased insulin sensitivity (Nickenig et al., 1997; Kurtz and Pravenec, 2004), and treatment with ARBs improves insulin resistance and diabetic complications (Henriksen et al., 2001; Igarashi et al., 2001; Kurtz and Pravenec, 2004). Studies in rats show that AT_1 receptor activation hinders insulin signaling upon infusion of AngII to cause insulin resistance (Patiag et al., 2000; Ogihara et al., 2002). Normally, insulin binding to insulin receptor enhances its tyrosine kinase activity and tyrosine phosphorylation of the insulin receptor substrates (IRS) and activation of phosphatidylinositol-3 kinase (PI3K). In rat VSMCs, AngII impairs coupling of the insulin receptor (IGF-1R) to PI3K and inhibits insulin-mediated IRS-1 tyrosine phosphorylation and association of IRS-1 with p85 (Folli et al., 1997). Alternatively, AngII increases serine phosphorylation of IRS-1 (Ser616 via ERK and Ser312 via JNK), thus interfering with insulin signaling (Andreozzi et al., 2004). Another mechanism described for interference with insulin signaling involves AngII-dependent tyrosine phosphorylation of PDK1 and ROS-sensitive Src activation (Taniyama et al., 2004; Taniyama et al., 2005). PKC is another kinase that may interfere with insulin signaling (Motley et al., 2003). Thus, AT_1 receptor signals may hamper insulin action at multiple levels. Hypertension and diabetes are frequently seen together,

indicating that interaction between AngII and insulin signaling plays an important role in cardiovascular pathology. ACE inhibitors are effective in the treatment of neuropathy found in diabetes (Malik et al., 1998; Malik, 2000). Coppey et al. (2006) showed that L-158809 (2-ethyl-5,7-dimethyl-3-[[4-[2-(2*H*-tetrazol-5-yl) phenyl]phenyl]methyl]imidazo[5,4-*b*]pyridine), an AngII receptor blocker, attenuated diabetic neuropathy in streptozotocin-induced diabetic rats. Until now, there are no clinical studies on the effect of ARBs in diabetic neuropathy, although the results suggest that ARB application may bear promise for treating neuropathy associated with vascular dysfunction and diabetic condition.

7. Angiogenesis and Cancer. AT_1 receptor–regulated cell proliferation and angiogenesis has biologic and therapeutic implications in cancer (Escobar et al., 2004; Deshayes and Nahmias, 2005; Uemura et al., 2005, 2006, 2011; Ino et al., 2011; Lau and Leung, 2011; Uemura and Kubota, 2012). Local AngII production is a proangiogenic stimulus in tumor microenvironment. Tumors implanted in wild-type mice developed intensive angiogenesis with vascular endothelial growth factor (VEGF) induction in tumor stroma. Systemic administration of an ARB reduced tumor-associated angiogenesis and VEGF expression in tumor stroma. In comparison, tumor-associated angiogenesis was reduced in $AT_{1a}R$ null mice, which was characterized by reduced expression of VEGF in the stroma and also reduced infiltration by macrophages. AngII-induced angiogenic factors production involves AT_1 receptor/JAK2/STAT3/SOCS3 signaling pathway. These results suggest that host stromal VEGF induction by AT_1 receptor is a key regulator of tumor growth and blockade of VEGF production by ARBs may be a novel therapeutic strategy against cancers (Egami et al., 2003; Fujita et al., 2005). AngII has been shown to function as a key role in neovascularization of hepatocellular carcinoma (Tamarat et al., 2002) in human breast carcinoma cells (Greco et al., 2002) as well as in invasive ductal breast cancer (Jethon et al., 2012). Both lisinopril and losartan treatment resulted in elevation in VEGF expression and angiogenesis, confirming the relationship between AT_1 receptor, VEGF, and vessel growth (Tamarat et al., 2002).

The choriocarcinoma cell proliferation is enhanced by AngII through the AT_1 receptor and activation of protein kinase C– and mitogen-activated protein kinase (Ino et al., 2003). AngII plays a role in the growth and chemoresistance of AT_1 receptor-positive pancreatic cancer cells through its action as a potent mitogen and antiapoptotic molecule (Amaya et al., 2004). AngII may promote prostate tumorigenesis via upregulation of PAX2 expression (Bose et al., 2009; Zhao et al., 2010). Thus, in the context of cancers of multiple tissues, AngII/AT_1 receptor signaling may favor tumor growth and may also contribute to aggressive etiology of cancers. Consideration of ARBs to restrict tumor growth should be a front line antitumor growth approach.

8. Autoantibodies and Malignant Hypertension. Autoantibodies that bind to and activate the AT_1 receptor exist in patients with hypertensive disorders and contribute to disease pathophysiology in preeclampsia, in kidney transplant recipients who develop refractory vascular rejection, and in patients with malignant hypertension (Roberts, 2000; Lodwick, 2001; Dechend et al., 2004; Herse et al., 2008; LaMarca et al., 2011; Herse and LaMarca, 2013; Xia and Kellems, 2013). More recently, AT_1 receptor–directed autoantibodies have been seen in patients with the autoimmune diseases, including systemic sclerosis, featuring autoimmunity, vasculopathy, and tissue fibrosis (Fu et al., 2000; Liao et al., 2002; Ansari et al., 2005; Dragun et al., 2005; Riemekasten et al., 2011). The AT_1 receptor autoantibodies found in preeclampsia, renal allograft rejection, and malignant hypertension are directed to an epitope, -AFHYESQ-, in the second extracellular loop of the AT_1 receptor (Wallukat et al., 1999). Treatment with AT_1 receptor blocker reverses the pathophysiological effects of AT_1 receptor autoantibodies in these diseases, suggesting the ability of autoantibodies to activate AT_1 receptors (Dechend et al., 2000; Dorffel et al., 2003). Autoantibody-induced production of reactive oxygen species (ROS) by the placenta and maternal tissues likely contributes to the oxidative stress associated with preeclampsia (Hubel, 1999; Many et al., 2000; Sikkema et al., 2001; Dechend et al., 2003; Thway et al., 2004). Injection of pregnant mice with IgG from preeclamptic patients leads to hypertension, proteinuria, and preeclampsia in mice (Zhou et al., 2008b). These features were prevented by an antibody-neutralizing, seven-amino-acid epitope peptide (Zhou et al., 2007, 2008a). The presence of AT_1 receptor autoantibodies was also confirmed in the experimental rat models of preeclampsia as extensively reviewed by Xia and Kellems (2013) recently.

III. The Angiotensin II Type 2 Receptor

Despite the controversial role it plays, the literature on the AT_2 receptor is quite extensive and confusing as reflected by 1061 peer-reviewed articles mined in our search (Fig. 1; Kemp, Karnik et al., 2014, http://www.guidetopharmacology.org/GRAC/ObjectDisplayForward?objectId=35.). The AT_2 receptor is a seven-transmembrane helical receptor that shares approximately 34% amino acid sequence homology with the AT_1 receptor. Its discovery in the 1980s as the dithiothreitol-potentiated or the PD123319-binding AngII receptor in vivo, cDNA cloning, identification of the gene, and generation of gene-knockout and transgenic mice in 1990s highlight the history of the AT_2 receptor (de Gasparo et al., 2000). The *AGTR2* gene is localized in the human chromosome Xq22-q23. Gene *Agtr2* in the rat is located on chromosome Xq34

and in mouse on chromosome at X12.5 cM. Gene organization is conserved in human and rodents, consisting of three exons, two introns, and the entire protein-coding frame contained in the third exon (de Gasparo et al., 2000). The first two exons contain a 5′-untranslated region (Vervoort et al., 2002). The AT_2 receptor is evidently different from the AT_1 receptor in terms of gene, genetic variations, protein sequence, tissue-specific expression, signaling mechanisms, regulation of receptor function, and pharmacological property. The in vivo physiologic functions of the AT_2 receptor are still not clearly defined. Fifteen years of research and >2500 publications devoted to AT_2 receptor delineate the discovery of unconventional ligand selectivity, agonist-independent signaling, and cGMP/NO signaling by AT_2 receptor. Identification of novel AT_2 receptor–interacting proteins and pharmacological agonists in recent years rekindled interest in this enigmatic receptor that could be therapeutically exploited for a possible protective role. This aspect of the AT_2 receptor is focused on in this review.

A. Structure

The human, rat, and mouse AT_2 receptor cDNAs encode a 363-amino acid protein that harbors hallmarks of a typical GPCR (Nakajima et al., 1993; Kambayashi et al., 1994; Koike et al., 1994). The AT_2 receptor amino acid residues show 72% divergence between rodents and humans, whereas the sequence is 99% conserved between rat and mouse. The AT_2 receptor encodes five potential *N*-glycosylation sites, which account for diverse molecular weights (68–113 kDa) observed in different tissues. Two potential disulfide bonds located in the AT_2 receptor extracellular region account for its characteristic DTT potentiation, which distinguishes it from the AT_1 receptor (Speth et al., 1991; Feng et al., 2000). The amino acid sequence identity between regions of the AT_2 receptor and AT_1 receptor varies between 24 to 34% in the transmembrane domain. The sequence of the third intracellular loop and the carboxyl terminal tail in AT_2 receptor diverge substantially from AT_1 receptor. These structural features of the AT_2 receptor form a potential basis for its poor coupling to G-proteins and lack of phosphorylation by GRKs as well as lack of desensitization after AngII binding. Although it was cloned over 15 years ago, little progress has been made with regard to solving the three-dimensional structure and identifying ligand-binding residues of the AT_2 receptor. The mechanism of ligand recognition and transmembrane signaling by the AT_2 receptor remains unexplored, which should be a priority in light of the possible protective role of the AT_2 receptor.

B. Pharmacology

Natural peptide hormone ligands AngII and AngIII bind the AT_2 receptor with nanomolar affinity and do not distinguish it from the AT_1 receptor. Although the semipeptide CGP42112 is an agonist for the AT_2 receptor, it is a nonspecific agonist for AT_1 receptor at high concentration (K_i 1.7 μM) (Brechler et al., 1993; Macari et al., 1994; de Gasparo et al., 2000). The analog, [*p*-amino-Phe^6] AngII is a classic ligand used to discriminate between AT_1 and AT_2 receptors. An additional potential endogenous agonist of the AT_2 receptor is vasoconstriction-inhibiting factor (VIF), which was isolated from human adrenal glands. VIF is a vasoregulatory peptide that modulates the vasoconstrictive effects of AngII by acting on the AT_2 receptor (Salem et al., 2015). VIF has been shown to inhibit AngII-induced phosphorylation of the p38 mitogen-activated protein kinase pathway but not of extracellular-regulated kinase 1/2 (Salem et al., 2015). A previously held view that AngI is a natural ligand for the AT_2 receptor is incorrect. Despite recognizing the same physiologic ligand, the pharmacophore for the AT_2 receptor is distinct from that of the AT_1 receptor (Miura and Karnik, 1999). Molecular recognition of AngII by the AT_2 receptor is "relaxed," operating upon a Lilliputian principle in that side-chain modifications of AngII that are detrimental to AT_1 receptor binding affinity are well tolerated by the AT_2 receptor. The discovery that interaction of no individual residue in AngII is critical for affinity of binding explains the ability of this receptor to engage analogs and metabolites of AngII (Miura and Karnik, 1999).

The nonpeptide antagonists PD123319 (ditrifluoroacetate) and PD123177 (trifluoroacetate salt) are widely used tools. PD123319 has a high affinity for the AT_2 receptor (K_i ~12 nM) and is approximately 10,000-fold more selective for AT_2 than AT_1 receptors. AT_2 receptor selectivity has been an important tool in defining the pharmacology and functions of this receptor in several different types of cells and tissues (Chiu et al., 1989; Chang and Lotti, 1990; Dudley et al., 1990; Wiest et al., 1991; Dudley and Summerfelt, 1993). Experimentally, the AT_2 receptor has been observed to bind a variety of ligands with an affinity order, CGP42112 > AngII ≥ AngIII > Compound 21 ≥ PD123319 >> AngIV > Ang (1-7) in the AT_2 receptor-transfected HEK-293 cells (Jones et al., 2011; Sipahi et al., 2011). Shorter angiotensin peptides may act as endogenous ligands at the AT_2 receptor; therefore, defining the physiology as well as the concept of separate receptors for AngII-metabolite peptides should formally rule out AT_2 receptor–mediated effects.

Research on the AT_2 receptor, specifically defining the beneficial effect, has long been hampered because of its low expression level in the adult and lack of ligands with pharmacological specificity. However, an AT_2 receptor–selective nonpeptide agonist, Compound 21 (Wan et al., 2004), and its application for defining in vivo function of this receptor is attracting attention to expound the distinct roles of the AT_2 receptor in many physiologic and pathophysiological states.

The AT_2 receptor is a new target for development of novel therapeutic agents to treat neuropathic pain. Human sensory neurons selectively express AT_2 receptor and not AT_1 receptor and hence may play a role in nociception (Anand et al., 2013; Smith et al., 2013a,b). In general, treatments for neuralgia and neuropathic pain are limited by poor efficacy and unfavorable side effects. The AT_2 receptor antagonist PD123319 and some analogs have been developed into orally active drugs for neuropathic pain (Smith et al., 2013a,b). EMA401 ((*S*)-2-(diphenylacetyl)-1,2,3,4-tetrahydro-6-methoxy-5-(phenylmethoxy)-3-isoquinolinecarboxylic acid), a highly selective AT_2 receptor antagonist, has been shown to inhibit capsaicin-evoked calcium influx in human and rodent sensory neuron cultures (Anand et al., 2013). In a phase-two clinical trial, EMA401 was found to provide superior relief of postherpetic neuralgia compared with placebo and was well tolerated by patients (Rice et al., 2014). EMA300 (5-[2,2-di(phenyl)acetyl]-4-[(4-methoxy-3-methylphenyl)methyl]-1,4,6,7-tetrahydroimidazo [4,5-*c*]pyridine-6-carboxylic acid), another small molecule antagonist of the AT_2 receptor, was found to alleviate neuropathic pain in mice with a chronic constriction injury of the sciatic nerve. Smith et al. (2013a,b) also showed that augmented Ang II/AT_2 receptor signaling in the dorsal root ganglia of chronic constriction injury rats was attenuated by EMA300, blocking p38 MAPK and p44/p42 MAPK activation and producing analgesia. Recently, EMA200 and EMA300 were assessed in a rat model of dideoxyxytidine-induced antiretroviral toxic neuropathy (ATN) (Smith et al., 2014). ATN is commonly observed in individuals infected with HIV and taking certain antiretroviral drugs to suppress viral replication. These individuals have a high prevalence of neuropathic pain and, therefore, a great need for new analgesics with $\geq$1000-fold selectivity over the AT_1 receptor. Administration of EMA200 and EMA300 induced dose-dependent analgesia in dideoxyxytidine rats, suggesting that these AT_2 receptor–specific analgesics should be investigated further for the relief of ATN (Smith et al., 2014). Further assessment of these and additional small molecule antagonists will advance the field of neuralgia management to develop promising therapeutics aimed at targeting the AT_2 receptor.

Based on the relaxed "conformation" hypothesis, AT_2 receptor was predicted to harbor high constitutive activity (Miura and Karnik, 1999). Several lines of evidence have indeed confirmed that AT_2 receptor functions in the absence of its ligand. The AT_2 receptor induces apoptosis in the absence of AngII stimulation, and this effect is not modulated by PD123319 (Miura and Karnik, 2000). Similarly, in neonatal cardiomyocytes, adenoviral-mediated AT_2 receptor expression induces myocyte growth, which is an effect not modulated by AngII, PD123319, or CGP42112 (nicotinic acid-Tyr-*N*-benzoxyl-carbonyl-Arg-Lys-His-Pro-Ile-OH) (D'Amore et al., 2005). In human coronary artery endothelial cells, lentiviral delivery of the AT_2 receptor changes expression of a large number of genes without AT_2 receptor ligands, and many fewer genes were differentially expressed when the AT_2 receptor–specific ligand CGP42112 was added (Falcon et al., 2005). Kemp et al. (2014b) found that AT_2 receptor expression antagonized regulation of microRNAs by AT_1 receptor and AngII stimulation of AT_2 receptor affected expression of only a few microRNAs, whereas the same treatment caused a robust response from AT_1 receptor. These findings suggest that altered expression of AT_2 receptor itself is a stimulus for function and that many cellular effects of AT_2 receptor expression are not contingent on ligand interaction with this receptor.

C. Mouse Models

Mouse models of AT_2 receptor deletion and overexpression have been useful tools for researchers to dissect the role of this receptor in cardiovascular and renal disease states. AT_2 receptor null mice have increased blood pressure compared with wild-type animals along with a host of other consequences, including increased sensitivity to injected AngII, attenuation of exploratory behavior, delay in VSMC differentiation, and increased susceptibility to renal-tubular developmental disease (Hein et al., 1995; Ichiki et al., 1995; Ichihara et al., 2001).

There are no gross developmental abnormalities in AT_2 receptor null mice, but blood pressure was found to be either unchanged (Hein et al., 1995) or increased (Ichiki et al., 1995) in these AT_2 receptor–null mice. Based on blood pressure elevation and augmented vascular sensitivity to AngII observed in the AT_2 receptor–null mice, AT_2 receptor action in vasculature has been suggested to be protective, counteracting blood pressure regulation by the AT_1 receptor (Hein et al., 1995; Ichiki et al., 1995). Several mechanisms may contribute to the protective effect, including regulation of AT_1 receptor expression (Tanaka et al., 1999), vascular bradykinin, and cGMP/NO production (Padia and Carey, 2013). The changes in exploratory behavior and greater stimulation of dipsogenesis in the AT_2 receptor null mice suggest neurologic dysfunction (Hein et al., 1995; Ichiki et al., 1995). The AT_2 receptor is prominently expressed in distinct brain areas such as the locus coeruleus (Rowe et al., 1990) and the amygdaloid nucleus (Song et al., 2002). Using riboprobe in situ hybridization histochemistry, Lenkei et al. (1997) mapped the distribution of AT_2 receptor mRNAs in the adult rat and found a predominant expression in the brain regions of the subfornical organ, the hypothalamus, and the lateral septum. They reported very limited overlap between the brain expression of AT_{1A} and AT_2 receptor mRNAs. de Kloet et al. (2014) used bacterial artificial chromosome transgenic AT_2 receptor-enhanced green fluorescent protein (eGFP) reporter mouse to localize AT_2 receptors at a cellular level. The authors examined for colocalized eGFP and AT_2 receptor mRNA within the

brain. This mapping approach localized AT_2 receptors to neurons within the nucleus tractus solitarius and median preoptic nuclei that regulate blood pressure, metabolism, and fluid balance, as well as limbic and cortical areas known to impact stress responding and mood. The paraventricular nucleus of the hypothalamus did not display AT_2 receptor-eGFP neurons, but efferent neurons terminating in the paraventricular nucleus and the GABA neurons surrounding the paraventricular nucleus did. The authors concluded those central AT_2 receptors are positioned to regulate blood pressure, metabolism, and stress responses in their transgenic mouse model.

Behavioral effects reported in AT_2 receptor null mice may be due to loss of AT_2 receptor regulation in these areas. Although developmental apoptosis of mesenchymal cells is not altered in AT_2 receptor null mice, an increased risk for renal diseases has been observed in AT_2 receptor null mice (Kakuchi et al., 1995). The massive expression of AT_2 receptor in the mesenchymal tissues was previously thought to regulate ontogeny of metanephros and tubulogenesis during development through apoptosis. However, nephrogenesis is not significantly altered by the AT_2 receptor null condition. More detailed and long-term follow up studies have demonstrated that the loss of the AT_2 receptor in mice may cause congenital anomalies of the kidneys and urinary tract (CAKUT syndrome) with a $>23\%$ penetrance.

Deletion of the AT_2 receptor in mice was also observed to cause inhibition of pressure natriuresis, vascular hypertrophy, and exacerbation of HF (Gross et al., 2000; Brede et al., 2001; Adachi et al., 2003). Renal vascular differentiation and the vascular smooth muscle contraction was altered because of the delayed expression of caldesmon and calponin, suggesting that the AT_2 receptor enhances the differentiation of VSMCs and therefore plays an important role in vasculogenesis (Yamada et al., 1998). Role of AT_2 receptor in cardiac, renal, and adrenal function is still unclear; its role in modulating pressure natriuresis is controversial as well (Keiser et al., 1992; Lo et al., 1995; Siragy and Carey, 1996). Pharmacological modulation of AT_2 receptor with agonists or antagonists in rats suggested an antidiuretic and antinatriuretic function of the AT_2 receptor. In rats and mice, AT_2 receptor activation by the selective agonist C21 induced pressure natriuresis and lowered blood pressure (Kemp et al., 2014a). However, studies in AT_2 receptor-knockout mice indicated exactly the opposite effects (Siragy, 2010).

Cardiac overexpression of the AT_2 receptor in mice did not cause obvious morphologic or functional changes, but AngII infusion decreased blood pressure and produced a negative chronotropic effect (Masaki et al., 1998). Stimulation of bradykinin activity and nitric oxide production after inhibition of the Na^+/H^+ exchanger in AT_2 receptor transgenic mice is attributed to this paradoxical phenotype (Tsutsumi et al., 1999).

Unequivocally defining the physiologic functions of the AT_2 receptor through transgenic and knockout mouse models did not occur. However, the findings with these models as well as physiologic explanations have been controversial and raise questions regarding a "yin-yang" paradigm invoked to explain the regulatory roles of the two AngII receptor types.

D. AGTR2 Genetic Polymorphism

In humans, gene polymorphisms that persist in populations and cause variable phenotypes in the individuals are widely studied. Genotyping humans for functional, single nucleotide polymorphisms (SNPs) within the *AGTR2* have been used in association studies to elucidate the pathogenic role of the AT_2 receptor in cardiovascular, neurologic, and renal diseases in various populations. Many of nonsynonymous changes have been associated with X-linked mental retardation (e.g., G21V, R324Q, I337V, and I53F). In addition, a base-pair deletion at position 395 causes a frame shift at Phe^{133} in the third transmembrane domain of the AT_2 receptor, resulting in a truncated protein, which is also associated with intellectual deficit (Vervoort et al., 2002; Bienvenu et al., 2003; Renieri et al., 2005). The effect of the +1675 G/A SNP has been associated with left ventricular structural changes in young men with arterial hypertension (Schmieder et al., 2001). In addition, the intronic polymorphism G allele in patients was reported to modulate left ventricular mass under high sodium intake (Ott et al., 2007). In hypertrophic cardiomyopathy patients, there is an association between this polymorphism and hypertrophy, which could potentially be used as a marker for genetic predisposition to left ventricular hypertrophy (Carstens et al., 2011). A second SNP, A/C 3123, has been linked to metabolic disorders such as blood pressure and body mass index among the diabetic population in Japan (Miyaki et al., 2006; Kotani et al., 2007). Further analysis showed that this SNP could be a marker for glycemic control (i.e., via HbA1c level) among Japanese women (Kotani et al., 2009). Finally, a C to A conversion at position 4599 was linked to preeclampsia associated with body mass index $\geq$25 kg/m (Dorn and Force, 2005), suggesting a gene-environment interaction (Zhou et al., 2013). Polymorphisms in intron 1 of the *AGTR2* gene (A-1332G) occur with higher frequency in human patients with congenital urinary tract abnormalities, suggesting that the AT_2 receptor may play an important role in the development of the urinary tract (Hohenfellner et al., 1999).

E. Signaling

The intracellular signal transduction processes activated by the AT_2 receptor, which govern cellular and physiologic responses, are atypical for a GPCR and distinctly different from those mediated by the AT_1 receptor (Fig. 7). Elucidating AT_2 receptor–specific signaling

pathways has been difficult and unsolved in most aspects, including receptor-proximal proteins that mediate the signaling by the AT_2 receptor.

1. G-Protein Involvement. All of the classic motifs and signature residues of a GPCR are present in the AT_2 receptor; however it fails to demonstrate classic features of G-protein signaling. Robust activation of heterotrimeric G-proteins, second messenger signals (calcium, DAG, cAMP, and IP_3), desensitization by phosphorylation, and receptor regulation by internalization is not observed in AT_2 receptor transfected cells (Kambayashi et al., 1993; Mukoyama et al., 1993; Miura and Karnik, 2002) or the native cells R3T3, PC12W, ovarian granulosa cells (Dudley et al., 1991; Pucell et al., 1991; Bottari et al., 1992a; Leung et al., 1992a; Webb et al., 1992). This behavior is consistent with observations of affinity of AngII binding, which is not affected by GTP or its analog GTPγS in tissues exclusively expressing the AT_2 receptor, such as human myometrium, rat adrenal glands, and bovine cerebellar cortex (Bottari et al., 1992a). Lack of agonist-induced desensitization and recycling of the AT_2 receptor is also observed in adult mice (Unger, 1999).

Multiple reports suggesting potential AT_2 receptor coupling to G_i/G_o have directly linked downstream signals to activation of this class of G-proteins (Fig. 7). For instance, AT_2 receptor s sensitive to GTPγS and pertussis toxin have been described in the locus coeruleus of the rat brain and thalamic or geniculate nuclei implying a coupling to heterotrimeric G-proteins, G_i and G_o (Tsutsumi and Saavedra, 1991). The AT_2 receptors appear to bind to $Gi_{\alpha 2}$ or $Gi_{\alpha 3}$ in developing fetus in rat (Zhang and Pratt, 1996) and in adult rat kidney cells. The intracellular third loop (ICL3) of the AT_2 receptor was shown to be important for its coupling to G_i (Hayashida et al., 1996). Evidence of ICL3 involvement was also shown in AT_2 receptor induction of apoptotic responses in the PC12W neuronal lineage cells (Lehtonen et al., 1999) and in AT_2 receptor–mediated inhibition of IP_3 generation in *Xenopus* oocytes (Kumar et al., 2002). More recent studies implicated the involvement of G_i in AT_2 receptor–dependent increases in nitric oxide synthase expression (Li et al., 2007a) and the inhibition of proximal tubule Na^+-ATPase by Ang(1-7) (Siragy and Carey, 1996; Siragy et al., 1996; Gohlke et al., 1998; Siragy, 2000). The production of prostacyclin in differentiated adipocytes was blocked by PD123177 and not by losartan, which suggests that this is an AT_2 receptor–mediated signal (Darimont et al., 1994). Whether this mechanism involved a G-protein, a G_i signaling pathway remains to be clarified. Direct G-protein activation assays in heterologous expression systems remain difficult with the AT_2 receptor. Whether ICL3 is important for coupling of the full-length receptor to G_i has not been validated by mutations in ICL3. Discrepant findings regarding G-protein coupling with the AT_2 receptor is an unresolved research challenge that constrains a full understanding of AT_2 receptor signaling mechanisms.

2. Protein Phosphatase Involvement. Depending on the tissues, activation of the AT_2 receptor can stimulate protein phosphatases (MKP-1, PP2A, SHP-1) and protein

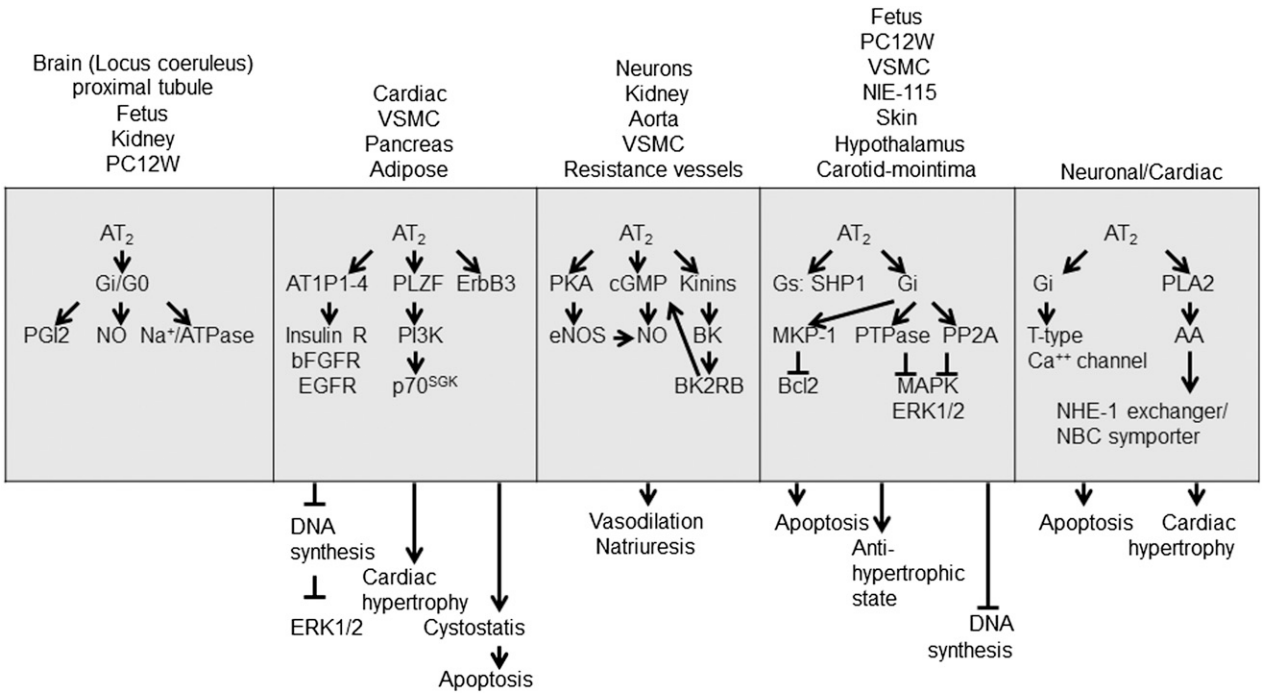

Fig. 7. Diverse signaling by AT_2 receptor. Each tile represents signaling pathways reported in different cellular and tissue context leading to physiologic or pathologic consequences. See main text for details.

dephosphorylation (Fig. 7). The activation of vanadate-sensitive tyrosine phosphatase as well as Ser/Thr phosphatases has emerged as a key mechanism accounting for the antigrowth and apoptotic effects of the AT_2 receptor (Bottari et al., 1992a; Horiuchi et al., 1997; Elbaz et al., 2000). The activation of the protein tyrosine phosphatase SHP-1 mediated by AT_2 receptor is pertussis toxin insensitive, thus a G_i-independent signal (Bedecs et al., 1997). SHP-1 coupling may actually involve an atypical G-protein scaffolding mechanism, $G_{\beta\gamma}$-independent constitutive association of the receptor with G_s and SHP-1 (Feng et al., 2002). In contrast to these studies, a G_i-coupled activation of PTP by AT_2 receptor in VSMCs was reported. Activation of G_i/G_o in these cells by a peptide corresponding to the third intracellular loop of the AT_2 receptor was inhibited by pertussis toxin, as well as by sodium orthovanadate a tyrosine phosphatase inhibitor. The serine/threonine phosphatase inhibitor okadaic acid did not block it (Hayashida et al., 1996). Similarly, activation of soluble SH-PTP1 to attenuate MAPK activation and DNA synthesis through thymidine incorporation observed in NIE-115 neuronal cells was suggested to be G_i/G_o dependent.

Activation of PTPases by the AT_2 receptor in cells may limit mitogen-activated protein kinase (MAPK) signaling and dephosphorylation of extracellular signal-regulated kinases 1 and 2 (ERK1/2) (Huang et al., 1995; Hayashida et al., 1996; Bedecs et al., 1997; Fischer et al., 1998; Akishita et al., 1999). The AT_2 receptor is a generalized negative regulator of intracellular kinase signaling, and thus it may antagonize growth stimulation by AngII, EGF, PDGF, or serum. AT_2 receptor knockout mice confirm this aspect, because elevated levels of ERK1/2 were reported at baseline and in response to serum (Akishita et al., 1999). ERK1/2 dephosphorylation may occur through three phosphatases: SHP-1, mitogen-activated protein kinase phosphatase 1 (MKP-1), and protein phosphatase 2A (PP2A) (Huang et al., 1995; Horiuchi et al., 1997; Yang et al., 1999). The AT_2 receptor–mediated suppression of MAPK activation in vessels is reported (Nakajima et al., 1995). The carotid artery expresses low levels of the AT_2 receptor (Viswanathan and Saavedra, 1992), but re-expressed AT_2 receptor at the edge of neointima during carotid injury or in a healing wound of the skin suppresses AngII-induced MAPK activity in a PD123319-sensitive manner to facilitate remodeling of these tissues (Nakajima et al., 1995). AT_2 receptor activation is linked to upregulation of SHP-1 in vascular-targeted AT_2 receptor transgenic mice (Matsubara et al., 2001). The AT_2 receptor in the neonatal hypothalamic neurons is reported to inactivate MAPK, and the presence of the AT_2 receptor antagonist PD123319 enhanced MAPK activity in these neurons. The ERK inactivated by the AT_2 receptor plays a physiologic role in vivo, depending upon the biologic context in which AT_2 receptor is engaged. For instance, in PC12W cells, which express only AT_2 receptor, AngII-regulated short-lived ERK phosphorylation plays a role in neuronal differentiation by AngII and nerve growth factor costimulation (Stroth et al., 2000). In the heart of AT_2 receptor transgenic mice, the antihypertrophic state of myocytes is associated with ERK dephosphorylation, which indicates that ERK inactivated by the AT_2 receptor plays a physiologic role in vivo (Masaki et al., 1998).

Conflicting reports also exist regarding phosphatase activation in AT_2 receptor–mediated proapoptotic effects. Dephosphorylation of Bcl-2 by MKP-1 was observed in AT_2 receptor–induced apoptosis in PC12W cells, which was ligand dependent (Horiuchi et al., 1997); apoptosis was shown to be a constitutive function of the AT_2 receptor that involves activation of p38 MAPK (Miura and Karnik, 2000). Overexpression of the AT_2 receptor in neonatal cardiomyocytes promoted growth (D'Amore et al., 2005). Overexpression and AngII stimulation of the AT_2 receptor in porcine cardiac fibroblasts inhibited protein tyrosine phosphatases (Warnecke et al., 2001). Observed contradictions further highlight the context-specific effects of the AT_2 receptor overexpression.

3. Scaffolding Protein Involvement. Choosing an unbiased approach to understanding how the AT_2 receptor suppresses cellular growth in different contexts yielded intriguing leads (i.e., yeast 2-hybrid "fishing" with the AT_2 receptor C-terminal tail as bait), which are indicative of completely novel signaling modalities (Fig. 7); however, these are in-progress studies that might lead to novel physiologic endpoints.

Negative regulation of several receptor tyrosine kinases (RTKs) including fibroblast growth factor, epidermal growth factor (Zhang et al., 2009) and insulin receptors by AT_2 receptor is described as transinactivation by AT_2 receptor activation of PTPases as well as inhibition of autophosphorylation of the RTKs (Elbaz et al., 2000; De Paolis et al., 2002). Possible mechanisms suggested for the transinactivation of RTKs include a direct physical interaction between the two receptors. The ErbB3 EGF receptor as an interacting partner with C terminus of the AT_2 receptor was identified. These authors also showed that replacing ICL3 of the AT_2 receptor with that of AT_1 receptor abolishes the interaction with ErbB3 and the inhibitory effects on cell proliferation and the activation of apoptosis (Pulakat et al., 1998, 2002).

The AT_2 receptor C-terminal tail interaction with the transcription factor promyelocytic zinc finger protein (PLZF) is one of the most surprising discoveries. After AngII stimulation, PLZF translocates from the cytosol to the plasma membrane and then promotes internalization of the AT_2 receptor with PLZF, leading to accumulation of AT_2 receptor in the perinuclear membrane. PLZF enters the nucleus where it activates the p85α subunit gene of PI3K and enhances $p70^{S6}$ kinase activity, which is essential for protein synthesis

(Senbonmatsu et al., 2003). The finding that AT_2 receptor internalizes when associated with PLZF is an interesting phenomenon, given that AT_2 receptor does not normally internalize (Mukoyama et al., 1995; Turu et al., 2006). PLZF is a highly expressed transcription factor in the heart; however, its expression is not affected in the AT_2 receptor–knockout mice. AT_2-knock–out mice do not show a hypertrophic response to pressure overload after aortic banding, this phenotype is associated with a failure to upregulate $p70^{S6}$ kinase after pressure overload. The AT_2 receptor–null hearts also fail to activate p85α transcription in response to AngII infusion. These data strongly suggest that the AT_2 receptor-PLZF-p85α-$p70^{S6}$ kinase–signaling axis might be important in the induction of cardiac hypertrophy (Senbonmatsu et al., 2000).

Two independent groups identified ATIP/ATBP50 (AT_2 receptor interacting protein) (Nouet et al., 2004; Wruck et al., 2005). At least four members are known to date for the ATIP-family (ATIP1-4). They all conserve an AT_2 receptor–interacting domain and can inhibit insulin, epidermal growth factor, and basic fibroblast growth factor-induced ERK1/2 activation and DNA synthesis in CHO cells in a similar manner to AT_2 receptor (Nouet et al., 2004). The ATIP-mediated inhibition of ERK1/2 requires AT_2 receptor expression but not treatment with AngII (Nouet et al., 2004). Li et al. (2007b) showed that siRNA-mediated knockdown of ATIP inhibits AT_2 receptor–mediated expression of methane methylsulfonate–sensitive 2, which plays an important role in the ubiquitin proteasome system and DNA repair. Methane methylsulfonate–sensitive 2 expression is increased in occluded/remodeling cerebral arteries, which is consistent with a protective role for the AT_2 receptor in brain injury. The siRNA-mediated knockdown of ATIP is also reported to reduce cell surface expression of the AT_2 receptor and suppress the antiproliferative effect (Wruck et al., 2005).

Although these studies yielded novel and interesting directions to pursue, the rationale for choosing the AT_2 receptor C-terminal tail as bait is not fully justified. There is no evidence to suggest that the AT_2 receptor C-terminal tail mediates the functions ultimately described for each scaffold protein. Rather surprisingly, involvement of $G_s/G_i/G_o$ proteins and the ICL3 appear to be an important determinant of AT_2 receptor coupling to PLZF as well as for interactions with ErbB3. These findings again point to G-protein requirement at least in unconventional mode and raise doubts about the mechanisms of scaffold signaling by the AT_2 receptor .

4. Nitric Oxide/cGMP Involvement. Nitric oxide (NO)–stimulated soluble guanylyl cyclase catalytic activity generates cGMP to generally exert protective effects in various tissues (Toda et al., 2007). Initial in vitro studies in neuronal cell lines suggested that AngII via the AT_2 receptor reduced cGMP levels in neuronal cells (Sumners and Myers, 1991; Sumners et al., 1991; Bottari et al., 1992b; Brechler et al., 1993). In other in vitro studies cGMP was not detected (Leung et al., 1992b; Webb et al., 1992; Mukoyama et al., 1993, 1995; Siragy and Carey, 1996). The studies by Liu et al. (1997) demonstrated that beneficial effects of AT_2 receptor involved kinin stimulation and cGMP production. Gohlke et al. (1998) also showed that AT_2 receptor stimulation increased cGMP levels in the rat aorta, which was further corroborated in AT_2 receptor–null mice studies (Siragy et al., 1999). The AT_2 receptor transgenic mice have elevated levels of cGMP in the aorta (Tsutsumi et al., 1999).

Whether the AT_2 receptor directly couples to NO/cGMP system especially in AT_2 receptor transgenic and knockout mice or through an indirect bradykinin B2-receptor dependent mechanism has been controversial. Tsutsumi et al. (1999) showed that the AT_2 receptor stimulates bradykinin production in VSMCs, and AngII-mediated cGMP response could be blocked with the bradykinin receptor antagonist. AT_2 receptor stimulates bradykinin production and promotes the NO/cGMP pathway in a paracrine manner, and this mechanism is important in AT_2 receptor–mediated vasodilatation of human coronary arteries (Batenburg et al., 2004). Different signaling circuitry may be involved in AT_2 receptor–dependent NO/cGMP production. In the thoracic abdominal aortic constriction mice model, AT_2 receptor activation induces phosphorylation of eNOS via a PKA-mediated signaling pathway (Yayama et al., 2006). Bradykinin acting on the bradykinin B_2 receptor can also induce PKA-dependent phosphorylation of eNOS. Evidences for a functional heterodimerization of the AT_2 receptor and B_2R receptors have been reported, which suggest that these receptors may physically associate and possibly increase NO production (Abadir et al., 2006).

5. Ion-Channel Protein Involvement. A highly reproducible signal from the AT_2 receptor seems to be coupling to ion-channel proteins (Fig. 7), such as the hypothalamic neuronal delayed rectifier potassium channel (Kang et al., 1993, 1994), the T-type calcium channel (Buisson et al., 1992, 1995) in other cells, which can suppress or induce cellular growth and differentiation (Nakajima et al., 1995; Stoll et al., 1995; Laflamme et al., 1996; Meffert et al., 1996; Munzenmaier and Greene, 1996; Tsuzuki et al., 1996a,b; Gallinat et al., 1998; Stroth et al., 1998; Cote et al., 1999; Gendron et al., 1999) as well as support apoptosis (Yamada et al., 1996; Chamoux et al., 1999; Gallinat et al., 1999).

Cardiac ventricular myocytes express both the AT_1 receptor and AT_2 receptor. Expression of AT_1 receptor along with AT_2 receptor is increased in the hypertrophic ventricles of SHR and two-kidney one-clip hypertensive rats (Suzuki et al., 1993; Busche et al., 2000). AngII-stimulated arachidonic acid production in cardiac myocytes is fully blocked by the AT_2 receptor antagonist PD123317, suggesting that AT_2 receptor mediates the activation of phospholipase A2 (Lokuta et al., 1994). The

AT_2 receptor–arachidonic acid signal activates the Na/HCO symporter system, which maintains pH in myocytes. The arachidonic acid–induced symporter activation in the heart is a unique function of the AT_2 receptor (Sandmann et al., 1998). In rats after myocardial infarction, the postinfarct upregulation of the Na1/H1-exchanger (NHE-1) and NBC ion transporter systems are differentially regulated by AT_1 and AT_2 receptors. ACE inhibitors prevent this regulation in heart tissue. However, the AT_1 receptor antagonists block NHE-1 upregulation, and the AT_2 receptor antagonists block the increase of the NBC (Horiuchi et al., 1999; de Gasparo et al., 2000; Nouet and Nahmias, 2000). This difference may be important in evaluating therapeutic intervention.

6. Involvement of Constitutive Activity. Ligand-independent actions of the AT_2 receptor (Miura and Karnik, 2002) is recognized as an important mode of its in vivo function in several scenarios. Upregulated AT_2 receptor expression as seen in remodeling adult tissues and developing fetal tissue can initiate constitutive signal transduction without AngII stimulation, leading to apoptosis (Miura and Karnik, 2000). Stimulation of apoptosis in prostate cancer cells was mediated by increased expression of the AT_2 receptor (Li et al., 2009b). AT_2 receptor gene transfer in the same cells mediated increased expression of bradykinin and iNOS in VSMCs. Downregulation of the AT_1 receptor was also observed by the same treatment (Jin et al., 2002). In another gene transfer study, Porrello et al. (2009) found that the AT_2 receptor constitutively antagonized AT_1 receptor–mediated cardiomyocyte autophagy. Homooligomerization of the AT_2 receptor through disulfide exchange between Cys^{35} in one AT_2 receptor and Cys^{290} in its dimerization partner was shown to be important for induction of apoptosis without AngII stimulation (Miura et al., 2005). Augmented release of bradykinin in mouse coronary artery endothelial cells was constitutive function (Zhu et al., 2010). Repression of ERK activity mediated by ATIP requires AT_2 receptor expression but not activation by AngII (Nouet et al., 2004). Feng et al. (2002) provided a potential mechanism for the constitutive function of the AT_2 receptor. They documented that constitutive association of G_α with SHP-1 and the AT_2 receptor is $G_{\beta\gamma}$ independent and this association is essential in AT_2 receptor-mediated ITIM-independent activation of SHP-1.

At the cellular level, a clear mechanistic model of AT_2 receptor signal transduction and cell physiology has yet to emerge. Despite intensive investigation, the AT_2 receptor is one of the inadequately understood components of the renin-angiotensin system.

F. Expression and Regulation

Expression of the AT_2 receptor is regulated in vivo in response to environmental cues. Both transcriptional and translational regulation seems to take place, but low AT_2 receptor expression levels are maintained in normal nongrowing cells. Growth factors, inflammatory mediators, and the growth phase of the cells regulate the AT_2 receptor gene. Extended serum depletion combined with insulin or IGF-1 or interleukin-1b (IL-1b) stimulate the expression of AT_2 receptor in VSMCs, whereas growth factors, like PDGF and phorbol ester, inhibit expression of AT_2 receptor (Kambayashi et al., 1993, 1996). The plasma insulin concentrations regulate AT_2 receptor expression in aorta (Kambayashi et al., 1996). Tissue-specific expression of the AT_2 receptor has been traced to enhancer elements in the AT_2 receptor gene promoter consisting of the AP-1 (inhibitor), C/EBP, NF/IL-6, IRS, and interferon regulatory factor IRF-2 (activator).

1. Developmental Regulation. Human tissue expression of AT_2 receptor has been documented by immunocytochemistry, reverse transcriptase–polymerase chain reaction, and microarray analyses (Ichiki and Inagami, 1995; de Gasparo et al., 2000; Herradon et al., 2004; Kim et al., 2005b; Chakrabarty et al., 2008; Petracco et al., 2012). In most tissues, the AT_1 receptor and AT_2 receptor coexist. The AT_2 receptor mRNA expression analysis provided a better picture of the tissue- and species-specific distribution (Kakuchi et al., 1995; Shanmugam et al., 1995; Johren and Saavedra, 1996). The AT_2 receptor is abundantly and ubiquitously expressed in the developing rat fetus and neonatal tissues, which lead to early speculation of a developmental role for the AT_2 receptor (Suzuki et al., 1993; Bastien et al., 1996).

In mouse fetal mesenchymal cells, >95% of the AngII binding is due to the AT_2 receptor. Regulation of its expression in relation to fetal developmental stages may suggest a potential role for this receptor in developmental processes. AT_2 receptor expression levels increase at embryonic days E11–E13. Maximal AT_2 receptor expression levels are seen on E19 followed by a rapid decline in the newborn animals to undetectable expression. The AT_2 receptor–null genotype has only a marginal effect, if any, on the development of mesenchyme-rich tissue or organs in the mouse such as skin, tongue, kidney, or adrenal (Kakuchi et al., 1995). However, a high incidence of urological abnormalities (Graham et al., 1997) and delay in vasculogenesis was reported in AT_2 receptor null mice (Yamada et al., 1998). The AT_2 receptor is the exclusive AngII receptor in the fetal rat aorta, which decreases by ~85% at 2 weeks of age and to 25% at 8 weeks of age (Viswanathan and Saavedra, 1992). AT_2 receptor blockade with PD123319 from E16 to E21 significantly decreases DNA synthesis in the developing aorta (Nakajima et al., 1995), and the AT_2 receptor–null mice have reduced levels of the VSMC differentiation markers calponin and caldesmon up to 4 weeks after birth (Yamada et al., 1999). In a similar fashion, stimulating the AT_2 receptor induces neurite outgrowth and regulates neurofilament expression in neural cells (Laflamme et al., 1996) as well as

promotes apoptotic effects neuronal cell lines (Yamada et al., 1996; Horiuchi et al., 1997). Increase in neuronal cell number in certain brain structures associated with central neurologic abnormalities, learning, and memory deficit have been reported for AT_2 receptor knockout mice (Hein et al., 1995; Ichiki et al., 1995; von Bohlen und Halbach et al., 2001). Whether the increase in cell number in these mice is due to increased neuronal proliferation or a suppression of apoptosis is not clear. The proapoptotic effect of the AT_2 receptor is not found in the cardiomyocytes of AT_2 receptor transgenic mouse (Sugino et al., 2001).

2. Adult Tissue Regulation. Receptor expression declines after birth, but the AT_2 receptor is expressed at low levels in the normal adult cardiovascular system, adrenal gland, kidney, brain, uterine myometrium, and skin. The capacity for regulated changes in expression of AT_2 receptor is retained in many adult tissues and often in remodeling tissues, a finding that suggests a potential role for this receptor in human cardiovascular disease as well.

The expression of both AT_1 receptor and AT_2 receptor in the adrenal gland, nonpregnant uterus, ovarian follicular granulosa cells, and cerebellar cortex is conserved in all mammals including humans (David, 1976; Whitebread et al., 1989; Criscione et al., 1990; Bottari et al., 1991; Pucell et al., 1991; Cox et al., 1993). There are some examples of tissues that contain more AT_2 receptor than AT_1 receptor. In the uterus, for example, expression of AT_2 and AT_1 receptors changes reversibly depending on pregnancy and parturition in human and sheep (Cox et al., 1993). The expression switching from high AT_2 receptor levels to a low-level AT_2 receptor and to completely AT_1 receptor is a fascinating developmental phenomenon that is not clearly understood.

The AT_2 receptor density increases in tissues under pathologic conditions in which inflammation and tissue remodeling occur. Such changes have been observed in tissues, such as skin wound, balloon-catheterized vasculature, infarcted myocardium, and ischemic brain during pathogenesis of hypertension, atherosclerosis, diabetes mellitus, nephropathy, and pulmonary fibrosis (Booz and Baker, 1996; Lemarie and Schiffrin, 2010). The observed increase in AT_2 receptor expression during pathology can be viewed as fetal gene reactivation, which is a prominent feature of various disease states, and this response may indicate that the AT_2 receptor has a definitive regulatory function in these instances (Paul et al., 2006). In rodents, AT_2 receptor expression is upregulated in HF (Ohkubo et al., 1997; Steckelings et al., 2005) and is up- and downregulated in a temporally dependent manner postinfarction (Nio et al., 1995; Lax et al., 2004). Expression of AT_2 receptors in humans is less clear. A decrease in AT_2 receptor expression during HF was reported (Matsumoto et al., 2000), whereas, an increase in expression was seen during atrial fibrillation (Goette et al., 2000). Myocardial AT_2 receptor expression is upregulated in patients with dilated cardiomyopathy. In the nonfailing human heart ~40% of AngII receptors are AT_2 receptor (Tsutsumi et al., 1998), and the AT_2 receptor constitutes 50–70% of angiotensin binding sites in the adult myocardium in humans (Regitz-Zagrosek et al., 1995). In the adult rodent myocardium, ~10% of adult cardiomyocytes express AT_2 receptor (Busche et al., 2000; Steckelings et al., 2005), but AT_1 receptor blockade increases AT_2 expression levels in rodents. Hence, under conditions of AT_1-inhibitor therapy, circulating AngII levels increase, creating the potential for increased AT_2 receptor function and facilitation of therapeutic tissue remodeling. This is the basis for targeting AT_2 receptors for therapy in patients treated with AT_1 receptor blockers to treat hypertension and HF. Adult brain AT_2 receptor may regulate central effects of angiotensin peptides in osmoregulation, cognitive functions, and behavioral functions (Hein et al., 1995; Hohle et al., 1995). Additionally, the role of the AT_2 receptor in regeneration and protection of neuronal tissue has been suggested (Steckelings et al., 2011a).

In the vasculature, a ratio of 80% AT_1 receptor to 20% AT_2 receptor exists in coronary endothelial cells derived from spontaneously hypertensive rats (SHR) (Stoll et al., 1995). Adult vasculature has low levels of AT_2 receptor, which may increase the sensitivity of vasculature to AngII when an AT_2 receptor-specific antagonist is present via the vascular AT_1 receptor (Verlander et al., 2011). The inhibitory effect of the AT_2 receptor on neointima formation after balloon catheterization of carotid artery has been extensively studied. The growth of the neointima was reduced by the AT_2 receptor antagonist PD123319 (Nakajima et al., 1995). Chronic AngII infusion increased the wall thickness-to-lumen ratio of mesenteric arteries associated with increase of both AT_1 receptor and AT_2 receptor (Cao et al., 1999). End-stage ischemic heart disease and dilated cardiomyopathy increased the density of the AT_2 receptor in endocardial, interstitial, perivascular, and infarcted regions of the ventricle of patients. That AT_2 receptor s may play a role in vessel formation is suggested by rich microvessels found in the border zone between noninfarcted and infarcted myocardium (Wharton et al., 1998).

In the kidney, 10 to 20% of total AngII receptors are AT_2 receptor, and no AT_2 receptors are detected in the rat renal cortex and glomeruli. Dietary sodium may modulate expression levels of AT_2 receptor in glomerular and interstitial tissue. The AT_2 receptor is present in large preglomerular vessels of the renal cortex and in the tubular interstitial cells in human kidney (Chansel et al., 1993; Goldfarb et al., 1994; Kaufman et al., 1999; Carey et al., 2001).

G. Pathophysiological Aspects of AngII Type 2 Receptor Activation

In the last 10 years, strides have been made toward understanding the pathologic and physiologic roles of

the AT_2 receptor (Knowle et al., 2000; Miura and Karnik, 2000; De Paolis et al., 2002). The AT_2 receptor has been implicated in cardiovascular disease as a beneficial moiety due to its proposed functions that, overall, appear to counterbalance the actions of the AT_1 receptor (Berk, 2003; Miura et al., 2010). Activation of the AT_2 receptor inhibits autophagy mediated by the AT_1 receptor in cardiomyocytes (Porrello et al., 2009). Overall, the AT_2 receptor may play a role in several AT_1 receptor–independent biologic processes as well (Ruiz-Ortega et al., 2000, 2001a; Mifune et al., 2000; Weidekamm et al., 2002; Benndorf et al., 2003; Gingras et al., 2003; Caballero et al., 2004; Zhao et al., 2005b; Zhu et al., 2000; Mertens et al., 2010). Notably, activation of the AT_2 receptor in cells of neuronal origin, induced neurite outgrowth, and elongation mediated cellular excitability and migration and, in some cases, caused neuronal cell death (Guimond and Gallo-Payet, 2012). Taken together, it has become clear that the signaling mechanisms of the AT_2 receptor are diverse and require further studies to fully elucidate the role of this receptor in biologic processes.

1. Regulation of Vascular Response. Physiologic effects of AT_2 receptor function in the vasculature are complex and can be both vasoconstricting and vasodilatating depending on the context. AT_2 receptor activation causes vasodilatation in most isolated arteries and it exerts a vasodepressor effect in vivo, when a concomitant, low-dose AT1R blockade is also administered (Widdop et al., 2003). Actions of the AT_2 receptor antagonist PD123319 and agonist CGP42112 consistent with this expectation have been demonstrated in rodent arteries as well as in human coronary microarteries (Carey et al., 2001; Batenburg et al., 2004). The pressor response to AngII is greater in AT_2 receptor–null mice than normal controls. VSMC-targeted overexpression of AT_2 receptor in transgenic mice causes vasodilatation in vivo (Tsutsumi et al., 1999). That the AT_2 receptor also mediates vasoconstriction in mesenteric resistance arteries of SHR and senescent rats is a recent finding (You et al., 2005). Antihypertensive treatment of 4 weeks restored AT_2 receptor expression and vasodilator function in SHR resistance arteries. Pinaud et al. (2007) reported that the AT_2 receptor induces a vasodilator effect in young rats and vasoconstrictive effects in old rats. Resolving the apparently conflicting roles of the AT_2 receptor in different vascular beds should be a focus for future studies.

2. Regulation of Cardiac Growth Response. The AT_1 receptor induces mitogenic effects in many tissues and cell types. Regulation of cell proliferation by the AT_2 receptor through an antiproliferative action opposed to the AT_1 receptor activation first discovered in coronary endothelial cells is also observed in microvascular endothelial cells, VSMCs, neuronal cells, pheochromacytoma cells, and fibroblasts (Stoll et al., 1995; Steckelings et al., 2005). The potential clinical ramifications of an AT_2 receptor-agonist therapy for the treatment of proliferative pathologies including cardiac fibrosis are vast. But the literature reports are confusing, for instance, AT_1 receptor–dependent stimulation of DNA and protein synthesis by AngII in neonatal cardiomyocytes and cardiac fibroblasts is observed only when the AT_2 receptor–selective antagonist PD123319 is present, suggesting that the cellular AT_1 receptor/AT_2 receptor ratio determines the outcome (Schorb et al., 1993; Crawford et al., 1994; van Kesteren et al., 1997). Increasing AT_2 receptor expression in neonatal cardiomyocyte cultures mediates myocyte hypertrophy independent of AngII (D'Amore et al., 2005), indicating that the AT_2 receptor is prohypertrophic. Studies of AT_2 receptor knockout and transgenic mice are ambiguous on the physiologic role of the AT_2 receptor in fibrosis. The myocardial perivascular fibrosis is increased in AT_2 receptor null mice (Wu et al., 2002), and an opposite outcome was observed in a different AT_2 receptor knockout mouse model (Kurisu et al., 2003). Overexpression of the AT_2 receptor in the heart significantly inhibited AngII-induced increases in perivascular fibrosis (Kurisu et al., 2003) in one model, whereas in another mouse model interstitial collagen increased (Yan et al., 2003b).

With regards to hypertrophy of myocardium, AT_2 receptor manipulation experiments in vivo yielded conflicting results. AT_2 receptor knockout prevents the induction of hypertrophy upon AngII infusion (Ichihara et al., 2001). However, transgenic mice overexpressing the AT_2 receptor in cardiomyocytes are normal and after AngII infusion they develop the same degree of hypertrophy as normal controls (Masaki et al., 1998; Moore et al., 2001; Yan et al., 2003b). Surprisingly the hypertrophic response to pressure overload is suppressed in AT_2 receptor–null mice, suggesting the AT_2 receptor is essential for hypertrophic process (Senbonmatsu et al., 2000). In a ventricular myocyte directed AT_2 receptor overexpression model, the AT_2 transgenic mice have an impaired Ca^{2+}-dependent inotropic response to AngII, associated with reduced activity of the Na^+/H^+ exchange (Nakayama et al., 2005). Transgene copy number influenced cardiac hypertrophy in this model. Hypertrophy under basal conditions was observed in MLC2v-AT_2TG mice with 18 copies of transgene with highest level of AT_2 receptor expression, whereas mice with 9 transgene copies did not display any signs of cardiac hypertrophy or HF (Yan et al., 2003b). Subjecting the 9 transgene copy mice to pressure overload by aortic banding significantly reduced left ventricular myocyte diameter, systolic pressure, and collagen compared with aortic-banded nontransgenic controls (Yan et al., 2003b). Thus, the role played by AT_2 receptor in heart is essential for both AngII-induced and pressure-overload myocardial hypertrophy, again raising doubts about the "yin-yang" paradigm that this receptor is a physiologic antagonist of AT_1 receptor actions.

3. Regulation of Fibrosis Response in Other Tissue. In vivo, PD123319 increases renal fibrosis, supporting the notion that the AT_2 receptor is anti-fibrotic in kidney as well. However, there are also conflicting reports that chronic PD123319 administration reduces collagen content in rats (Levy et al., 1996). Aggravated renal injury is observed in AT_2 receptor–knockout mice (Benndorf et al., 2009). The AT_2 receptor–deficient mice had exaggerated mortality accompanied with increased risk of albuminuria, renal fibrosis, glomerular injury, lymphocyte infiltration, and chemokine expression compared with renal ablation control mice. The blood pressure and RAS metabolites were similar in these groups.

Studies in disease models carrying the AT_2 receptor-null genotype show greater susceptibility for pathogenesis of cerebral infarction and atherosclerosis (Iwai et al., 2005). Angiotensin AT_2 receptor protects against cerebral ischemia induced neuronal injury (Li et al., 2005; Kaschina et al., 2008). In cerulean-induced pancreatitis, expression of AT_2 receptor is associated with pancreatic fibrosis, which is increased through a TGF-β–mediated mechanism (Ulmasov et al., 2009).

In the last 10 years, strides have been made toward understanding the role of the AT_2 receptor in various pathogenic models (Knowle et al., 2000; De Paolis et al., 2002; Miura and Karnik, 2002). The AT_2 receptor has been implicated in cardiovascular disease as a beneficial moiety due to its proposed vasodilatory role, ability to inhibit cell growth, induce apoptosis, and inhibit activation of MAPK functions that, overall, appear to counterbalance the actions of the AT_1 receptor (Berk, 2003; Miura et al., 2010). Overall, the AT_2 receptor may play a role in several AT_1 receptor–independent biologic processes (Mifune et al., 2000; Ruiz-Ortega et al., 2000, 2001a; Zhu et al., 2000; Weidekamm et al., 2002; Benndorf et al., 2003; Gingras et al., 2003; Caballero et al., 2004; Zhao et al., 2005b; Mertens et al., 2010). Furthermore, activation of the AT_2 receptor inhibits autophagy mediated by the AT_1 receptor in cardiomyocytes (Porrello et al., 2009). Notably, activation of the AT_2 receptor in cells of neuronal origin, induced neurite outgrowth and elongation, mediated cellular excitability, and migration and, in some cases caused, neuronal cell death (Guimond and Gallo-Payet, 2012). Taken together, it has become clear that the AT_2 receptor signaling mechanisms are diverse and require further studies to fully elucidate the role of this receptor in biologic processes.

H. Drug Targeting the AngII Type 2 Receptor

Wan et al. (2004) developed a highly selective, orally accessible nonpeptide AT_2 receptor agonist, called Compound 21 (C21). A potential therapeutic role for the AT_2 receptor is being propagated since the discovery of C21; it can confer acute vasorelaxation in vitro and has been shown to have organ protection effects in vivo. Specifically, C21 was observed to improve postmyocardial infarction (Kaschina et al., 2008) and provide vasodepressor effects in spontaneously hypertensive rats (Bosnyak et al., 2010). In addition, the ligand was shown to reduce myocardial fibrosis and vascular injury in hypertensive stroke-prone rats (Rehman et al., 2012). Controversy surrounds the use of C21 for therapeutic intervention, however. It was recently speculated whether the effects of C21 were AT_2 receptor specific or just off-target effects as others have cautioned (Guimond and Gallo-Payet, 2012; Verdonk et al., 2012). Therefore, development of additional AT_2 receptor–specific agonists and antagonists is crucial and will expand the field, supporting an important role for targeting of the AT_2 receptor in disease states.

The increased knowledge of the expression pattern and mechanisms of action of the AT_2 receptor has substantially contributed to understanding the role of AngII in physiologic and pathologic conditions. Synthesis of additional highly selective AT_2 receptor ligands will allow for the exploration of novel receptor-dependent effects in the cardiovascular, renal, and nervous systems. The ability of the AT_2 receptor to be a therapeutic target is promising and provides insight into the complexities of RAS.

IV. Angiotensin II Type 3 Receptor

In addition to AT_1 receptor and AT_2 receptor, the presence of an AT_3 subtype was reported by Chaki and Inagami (Chaki and Inagami, 1992a; Inagami et al., 1993). Unique pharmacological properties observed in mouse neuroblastoma cells (Neuro-2A) led to the designation of new subtype, AT_3 receptor. Binding affinity of this subtype is ~12.0 nM for [^{125}I]AngII, which was inhibited by AngII with a K_i value of ~7 nM. [^{125}I]AngII binding to this subtype was not inhibited by AngIII, the AT_1 receptor antagonist Dup753 or the AT_2 receptor antagonist PD123319. [^{125}I]AngII binding was insensitive to GTP analogs. This subtype expressed in differentiated Neuro-2A cells was shown to stimulate cGMP formation in these cells. The stimulation was blocked by the [Sar^1,Ile^8]AngII but not by Dup753 or PD 123319 (Chaki and Inagami, 1992b). A 2.2-kb full-length cDNA encoding a putative AT_3 receptor was cloned by screening an adrenal cortex library (Inagami et al., 1993). The putative AT_3 receptor cDNA encodes a 40-kDa protein with 95% amino acid identity to rAT_1 receptor. The overall nucleotide similarity is 71% because of low homology in the 5′ (58%)- and 3′ (62%)-untranslated regions. A different receptor subtype, rAT_{1C}, was reported to be 95% homologous to the rAT_3 receptor nucleotide sequence and less so to $rAT_{1\,A}R$ (90%) and rAT_1R (82%) subtypes. Expressed AT_3 receptor in COS-7 cells mediates agonist-induced calcium mobilization that is not inhibited by AT_1 receptor inhibitors. The AT_3 receptor mRNA is reported to be most abundant in the adrenal cortex and pituitary and differs from AT_1

receptor mRNA tissue distribution. The AT_3 receptor sequence contains two additional potential phosphorylation sites for protein kinase C (Sandberg et al., 1992). Despite these initial efforts, further characterization of putative AT_3 receptor has stalled. Furthermore, a gene for encoding the putative AT_3 receptor is not annotated despite the availability of a cDNA clone, possibly suggesting further uncertainty for an AT_3 receptor .

V. Angiotensin IV Binding Site(s)

Defined as novel "non-AT_1 and non-AT_2", angiotensin IV (AngIV: VYIHPF) binding entity associated with neuronal cognitive processes and renal vascular functions (Fig. 8) was initially nominated as the AT_4 receptor in 1995 (de Gasparo et al., 2000). A specific membrane-binding site for the [^{125}I]AngIV peptide was defined as the AngIV binding site (Swanson et al., 1992). This AngIV binding site was found to be concentrated in brain, heart, kidney, adrenals, and blood vessels (Wright et al., 1993, 2008). Functional distinction of AngIV binding to the AngIV binding site was demonstrated. AngIV analogs induced several marked biologic effects in central nervous, renal, pulmonary, and vascular tissues (Handa, 2001; Li et al., 2002; Vinh et al., 2008; Wright et al., 2008). Human LNPEP gene (*Lnpep* in rodents) product IRAP/AngIV binding site was identified and is believed to be the sole AngIV binding site until recently. Nearly 20 years of research only shows the complexity associated with the concept of a unique AngIV binding site in vivo that prevent unambiguously defining a receptor molecule that satisfactorily accounts for pharmacology and function as the AngIV receptor (the IUPHAR Nomenclature Committee, Karnik et al., 2014; http://www.guidetopharmacology.org/GRAC/FamilyIntroductionForward?familyId=6.).

A. Functional Definition of AngIV Binding Sites

Existence of a heat resistant receptor that reversibly bound [^{125}I]AngIV with high affinity ($K_d \approx 1$–5 nM) in bovine adrenal membranes was described (Harding et al., 1992; Jarvis et al., 1992; Swanson et al., 1992; Sardinia et al., 1993). This receptor did not bind the peptide analogs of AngII, [Sar^1] AngII, [Sar^1,Ile^8] AngII, [Sar^1,Ala^8] AngII, Ang(1–7), AngIII, and the nonpeptide inhibitors of AT_1 and AT_2 receptor Dup753 (losartan), PD123177, and CGP42112A (Harding et al., 1992; Swanson et al., 1992; Hanesworth et al., 1993). Histoautoradiographic mapping techniques determined the greatest concentrations of [^{125}I]AngIV specific binding sites in the brain. The brain distribution of AngIV binding sites was distinct from that of both AT_1 and AT_2 receptors. These criteria lead to the term AngIV binding site, which is by definition, linked to regulation of cognitive, sensory, and motor functions. These AngIV binding sites are not associated with functions where AT_1 receptor or AT_2 receptor play a predominant role in physiologic functions, such as water-electrolyte balance, cardiovascular regulation, and control of thirst behaviors. However, AngIV effects observed in the cardiovascular and renal tissue, which was initially thought to be mediated by the AngIV binding site, may actually be mediated through the AT_1 receptor. Therefore, definition of AngIV binding site excludes functions associated both AT_1 and AT_2 receptors.

B. Structure of the AngIV Binding Protein, Insulin Regulated Amino Peptidase

The putative AngIV binding site was biochemically identified as a ~160- and ~190-kDa glycoprotein on reducing SDS-polyacrylamide gel (Bernier et al., 1995a; Wright and Harding, 1995). The adrenal and bovine endothelial AngIV binding sites were reported as dimeric on nonreducing gels at 225 kDa (Bernier et al., 1998). Similarly, the AngIV binding site polypeptides from bovine heart, thymus, kidney, bladder, aorta, and hippocampus were dimers (Zhang et al., 1999). Soon after its characterization, AngIV binding site was thought not to be a GPCR because of its mobility on SDS gels as well as lack of GTPγS effect on [^{125}I]AngIV binding in rabbit heart, guinea pig brain, and rat vascular smooth muscle (Hall et al., 1993). That AngIV binding site is not a GPCR now seems to be unanimous.

By purifying and sequencing the putative AngIV binding protein, Albiston et al. (2001) identified it as insulin regulated aminopeptidase (IRAP, E.C. 3.4.11.3 also called LNEP for leucyl-*N*-exopeptidase). IRAP is a Type 2 transmembrane protein of the gluzincin aminopeptidase family (Rogi et al., 1996; Nomura et al., 2005). This family includes homologs such as aminopeptidases A and N (AP-N: endothelial cell 3.4.11.2). Reflecting distinct activities, the mammalian IRAP/AngIV binding site has many names, oxytocinase, cystinyl aminopeptidase, placental leucine aminopeptidase, gp-160, or vp-165, depending on its independent cloning (Vauquelin et al., 2002a; Albiston et al., 2004a; Nomura et al., 2005; Tsujimoto and Hattori, 2005). Human IRAP/AngIV binding site contains 1025 residues with N-terminal 110-amino acid-long intracellular domain, a 21-residue-long transmembrane segment (111–131), and an 871-residue-long extracellular C-terminal domain (132–1025), which contains the catalytic and zinc binding sites. The catalytic site is composed of a GXMEN motif that is crucial for the exopeptidase activity and includes the Zn^{2+}-binding motif HEXXH-X_{18}-E (Kandror and Pilch, 1994; Kandror et al., 1994; Keller et al., 1995; Ross et al., 1996). HEK 293T cells transfected with IRAP expression plasmid specifically bind the AngIV binding site ligands, AngIV and LVV-hemorphin 7, and compete for the binding of ^{125}I-[Nle^1] AngIV with IC_{50} values of 32 and 140 nm, respectively. Both immunohistochemistry and mRNA hybridization histochemistry analysis determined in parallel on the brain matched that of the AngIV binding site determined by radioligand binding.

IRAP/AngIV binding site

Gene and Protein: The gene for IRAP/AngIV binding site is LNPEP (EC 3.4.11, chromosome 5, gene ID: 4012). It is a single pass 1025 residue long Type II trans-membrane zinc protease. It is also known as insulin-regulated aminopeptidase (IRAP), Vasopressinase, placental leucine aminopeptidase (PLAP), oxytocinase (Otase), cystinyl aminopeptidase (CAP).

Functional Surrogates:
1. Aminopeptidase N (AP-N, EC3.4.11.2) a paralog of LNPEP involved in processing of peptide hormones, such as angiotensin III and neuropeptides and may act as a receptor. AP-N (chromosome 15, gene ID 290) is also known as CD13; LAP1; P150; GP150. The 967 amino acid protein with long extracellular carboxyl terminal domain contains a consensus zinc-binding sequence.

2. cMET/HGFR, is the proto-oncogene MET product hepatocyte growth factor receptor (EC 2.7.10.1, chromosome 7, gene ID 4233) is a receptor tyrosine-kinase, also known as HGFR; AUTS9; RCCP2; c-Met. Ligand Binding induces autophosphorylation that provides docking sites for downstream signaling molecules such as PI3-kinase or the adapter proteins leading to the activation of signaling cascades including the RAS-ERK, PI3 kinase-AKT, RAS-ERK pathways.

Tissue Distribution: Placenta, Uterus, Ovary, Brain, Smooth muscle, Heart, Kidney,Liver, Lung, skeletal muscle, Skin, Prostate, White blood cells and platelet.

Natural Ligands: Angiotensin 3-8 (AngIV), Vasopressin, Oxytocin, LVV-hemorphin, LVV-hemorhin-7.

Agonists: Norleucine1-angiotensin VI, Norleucine1-Leu3-psi(CH2-NH2)3-4-angiotensin IV , Lys-Bradykinin.

Antagonists/Inhibitors: WSU 3033, Divalinal-AngIV (Divalinal), Compound 8 [beta(3)-homotyrosine Ang IV], Compound 12 [beta(3)-homotyrosine Ang IV], Compound 7 [macrocyclic Ang IV], Aminopeptidase inhibitor 7B, Compound 19 [macrocyclic Ang IV] Ang IV-NH(2), IVDE77, AL-11.

Functional Assays: Memory retention, retrieval and reversal of induced-amnesia; Calcium Signaling; Enhanced synaptic long term potentiation (LTP) in dentate gyrus; Inhibition of neurohormone cleavage; Cerebral vascular relaxation and microcirculation; Ang IV dependent pulmonary endothelial NOS activation; Dopamine release in striatum.

Signaling: Intracellular calcium, PI3-kinase signaling; eNOS activation, MAPK activation, engage Tankyrase 1 and 2, Rab GTPase-activating protein As160.The signal transduction of AT_4R can be questioned since it is not unequivocally shown to be mediated by the binding of AngIV to IRAP. Recombinant expression of human IRAP in cell lines failed to establish ligand induced signaling.

Physiological Consequences of Altering Gene Expression: The *lnpep* -null mice maintained normal glucose uptake and distribution of Glut4 into muscle and fat cells.

The loss of spatial memory and AngIV binding site are age related in *lnpep*-null mice. AngIV-dependent fibrinolysis via PAI-1 and arterial thrombosis after vascular injury is attenuated in the *lnpep* -null mice. The *lnpep* -null mice are less sensitive to the development of acute pentylenetetrazol (PTZ)-induced seizures. The *lnpep* -null mice lack antidepressant-effect of oxytocin, less risk for ischemic brain damage and stroke analogous to the effect of Ang IV.

Fig. 8. IRAP/AngIV binding site. See main text for details and Supplemental Material.

The AngIV binding site ligands dose dependently inhibit the IRAP catalytic activity. In vivo, IRAP hydrolyzes the N-terminal amino acid from neuropeptides including arginine-vasopressin, cholecystokinin-8, dynorphin, met-enkephalin, lysine-bradykinin, neurokinin A, neuromedin B, oxytocin and somatastatin, (Herbst et al., 1997; Matsumoto et al., 2001; Lew et al., 2003). Accordingly, IRAP was proposed as the first AngIV binding site (see Fig. 8 and Supplemental Material).

Albiston et al. (2001) proposed that the AngIV binding site ligands may exert their effects by inhibiting the catalytic activity of IRAP and extending the half-life of its neuropeptide substrates to potentiate cognitive functions attributed to AngIV binding to the AngIV binding site. The LNPEP gene product (*Lnpep* gene in rodents) was considered the sole AngIV binding site until recently (Vauquelin et al., 2002a).

C. Pharmacology of AngIV Analogs

1. Agonists of the AngIV Binding Site. The characteristics of the AngIV molecule critical for high-affinity binding to the AngIV binding site in tissue include preservation of residues at positions 1, 2, or 3 of the AngIV, whereas positions 4, 5, and 6 can be replaced (Sardinia et al., 1993; Wright et al., 1995). A detailed study (see Supplemental Material) of position 1 of AngIV

revealed that high-affinity AngIV binding requires a primary amine in position 1 and L-hydrophobic residues at position 1 favor binding. Thus, L-norleucine1-AngIV resulted in a very high-affinity analog (K_d in the picomolar range). The CH_2-NH isostere substituted at 1–2 peptide linkage produced a high-affinity analog that increases rotation of peptide bond (Sardinia et al., 1993). An L-aromatic amino acid at position 2 and L-hydrophobic amino acid at position 3 of the AngIV molecule are critical to bind to AngIV binding site. Deletion of the C-terminal phenylalanine from of AngIV does not alter binding affinity, but this derivative is inactive in manipulating physiologic blood flow (Wright et al., 1995). The minimal AngIV-like peptide ligand to bind AngIV binding site with high affinity requires a sequence, Val^1-Tyr^2-Ile^3-R1-R2. An agonist requires a phenylalanine in addition at the C-terminal position, a structural feature of AngIV, which would also permit activation of signaling through the ubiquitously present AT_1 receptor. In most studies of AngIV analogs, inadvertent activation of the AT_1 receptor may have to be carefully ruled out. The peptide LVV-hemorphin-7 binds and activates the AngIV binding sites in vivo. LVV-H7 specifically displaced [^{125}I]AngIV binding to adrenal and brain membranes.

2. Antagonists of the AngIV Binding Site. Harding's laboratory made early strides overcoming the difficulties of design and synthesis of AngIV binding site antagonists. They had success with Val substitution coupled with reducing peptide bonds in the Val-Tyr-Val segment generating divalinal-AngIV (Sardinia et al., 1993; Sardinia et al., 1994; Krebs et al., 1996). A second antagonist with norleucine in position 1, leucine in position 3, and reduced peptide bonds at the first and third bond positions (Norleual-AngIV) was subsequently created and characterized. This analog binds the AngIV binding site with high affinity and has been reported to not bind AT_1 receptor and AT_2 receptor. Divalinal-AngIV prevented c-Fos expression, blood flow induced by AngIV in brain, as well as the inhibition of proximal tubule sodium transport by AngIV (Handa et al., 1998).

3. Inhibitors of the Insulin Regulated Amino Peptidase Activity. Concept of IRAP as the potential AngIV binding site stimulated development of newer and more selective analogs and resulted in the development of a new class of cognitive enhancers, which target the catalytic site of IRAP (Albiston et al., 2008) (see Supplemental Material). Efforts were taken to develop AngIV-derived IRAP inhibitors with improved selectivity versus AP-N and the AT_1 receptor, as well as resistance to proteolytic degradation. Lukaszuk et al., 2008) reported that the AngIV analog AL-11 (pK_i 7.25 for IRAP enzyme activity) in which the β-homo amino acid β^2hVal is responsible for stability and the β^3hPhe for selectivity. Further improvement was obtained by substitution of His^4-Pro^5 with a constrained Trp analog (Aia-Gly) to obtain AL-40 with a pK_i is 8.07 for IRAP (Lukaszuk et al., 2009). Substitution of the latter Gly^5 by norvaline led to an analog (IVDE77) with further increased affinity (pK_i is 8.77 for IRAP) (Lukaszuk et al., 2011; Nikolaou et al., 2013). The latter compound was also ^{3}H-labeled and characterized as a useful tool for the detection of IRAP/AngIV binding site under physiologic conditions, i.e., in the absence of any metal ion chelators (Nikolaou et al., 2013).

Synthesis of peptidomimetics targeting IRAP activity lead to successful replacement of the C-terminal His-Pro-Phe in AngIV replaced by small aromatic γ-turn mimetics (Compound 29, K_i = 26 nM). Compounds with low nanomolar inhibitory activity and enhanced selectivity for IRAP over the structurally related amino peptidase, AP-N, were obtained. Additional studies are needed to elucidate bioactive conformation for IRAP inhibition and presumably functional selectivity for AngIV binding site–mediated cognitive effects (Anderson et al., 2010, 2011; Axen et al., 2006). With a bioinformatics in silico screening approach, a large library of compounds against a structure homology of IRAP (Ye et al., 2008; Albiston et al., 2011) benzopyran compounds were identified, synthesized, and optimized; the highest affinity compound HFI-437 has a pK_i of 7.70 for IRAP. The latter nonpeptide inhibitors were found to enhance the performance of rats in different memory paradigms (Mountford et al., 2014).

Yang et al. (2008) used IRAP/AngIV binding site-selective ligands, LVV-hemorphin-7 and AT4-16, for intravenous, intrarenal, and intracerebroventricular infusion to discriminate AngIV effects through AT_1 and AngIV binding sites. They observed that AngIV had no effects on mean arterial pressure, renal blood flow, or urinary sodium excretion in the presence of IRAP inhibitors. In addition, they reported that the majority of the AngIV-sensitive aminopeptidase activity in kidney membranes is due to AP-N and not the IRAP/AngIV binding site. Some of the central and peripheral vasoactive effects of AngIV were mediated through the AngIV binding sites in other studies (Lew et al., 2003; Axen et al., 2007). Increase in blood pressure from chronic AngIV elevation in the brain was found to be AT_1 dependent in a transgenic mouse model, although specificity of action of AngIV in this model should be a question (Lochard et al., 2004). Thus, central and peripheral pressor and vasoconstrictor effects of AngIV are very likely mediated through AT_1 receptor in vasculature. The activation of the AT_1 receptor by AngIV is a well established pharmacological effect, but this effect is distinct from the memory-enhancing effects of AngIV (Yang et al., 2008). Stragier B. et al. (2006) independently demonstrated that AngIV and LVV-H7 did not significantly alter hippocampal blood flow as determined by laser Doppler flow measurement, excluding the vascular effects responsible for cognitive functions of either peptide (Jewett et al., 1991) (see Fig. 8 and Supplemental Material).

D. Signaling

1. Effects on Insulin Regulated Amino Peptidase Signaling as a Transmembrane Receptor. Prior to the identification of IRAP as AngIV binding site, the AngIV binding site was thought to be a multimeric, 225-kDa complex in bovine tissues (Bernier et al., 1995b; Zhang et al., 1999). It is now clear that AngIV binding sites are not coupled to G-proteins in tissues such as bovine endothelium, guinea pig brain, and rat vascular smooth muscle (Hall et al., 1992). The N-terminal domain of IRAP may participate in signal transduction with AngIV acting as an agonist in the activation of intracellular signaling, which may mediate the physiologic effects of AngIV. Vauquelin et al. (2002b) proposed that IRAP is a classic transmembrane receptor, with an intracellular tail that would interact with intracellular signaling molecules. This idea is based on the finding that AngIV interacts with a juxta-membrane domain of IRAP/AngIV binding site in bovine heart membranes. This putative binding site for AngIV is different from that of the active site of IRAP, which implies that AngIV binding may modulate transmembrane signal transduction by AngIV binding site and the IRAP activity by an allosteric mechanism (Caron et al., 2003). In the structurally related AP-N, binding of monoclonal antibodies activates IP3-linked increase of intracellular calcium and phosphorylation of MAPK (Navarrete Santos et al., 2000a,b,c). Expression of c-Fos is induced by intracerebroventricular infusion of AngIV in brain centers of rats, which are associated with cognition (Roberts et al., 1995). Several studies have shown that AngIV binding site ligands are capable of activating different intracellular signaling pathways. These include a variety of mechanism(s), including an increase of the intracellular calcium concentration, modulation of MAPK activation of NFκB signaling, increased cGMP production, and more downstream changes in DNA synthesis. It should also be noted that in no classic signaling could be demonstrated in CHO cells despite the presence of AngIV binding sites (Albiston et al., 2007). In pulmonary artery endothelial cells, AngIV binding to AngIV binding sites activates eNOS and stimulates cGMP accumulation and causes pulmonary arterial vasodilation (Patel et al., 1998). This effect was blocked by divalinal AngIV as well as by the phosphoinositide 3-kinase inhibitors (Patel et al., 1998). Induction of PAI-1 by AngIV has been implicated in the pathogenesis of renal interstitial fibrosis in several forms of chronic glomerulonephritides (Gesualdo et al., 1999). AngIV stimulates cell proliferation in rat anterior pituitary without requiring AT_1 receptor (Ptasinska-Wnuk et al., 2003) and also activates NFκB, leading to the transcription of proinflammatory genes, intercellular adhesion molecule-1, IL-6, MCP-1, PAI-1, and TNFα in VSMCs. These effects were blocked by treatment with AngIV binding site antagonist, suggesting that signal transduction activated by AngIV may underlie pathogenesis of cardiovascular diseases (Esteban et al., 2005).

IRAP inhibitors, such as divalinal-AngIV and LVV-hemorphin-7, which bind to the IRAP active site directly, produce similar effects. For instance, increasing the extracellular dopamine levels in the striatum of the rat by these inhibitors was comparable to AngIV. This finding suggests that active-site binding of the inhibitors mediates modulation of extracellular dopamine levels in striatum (Stragier et al., 2004). In a related study, the authors hypothesized that IRAP and/or AP-N possibly act as receptors for IRAP inhibitors and AngIV in mediating dopamine release in the striatum by receptor-mediated signaling (Stragier et al., 2007). High-affinity binding of AngIV to only the Zn-depleted IRAP apoenzyme may inhibit IRAP activity by stabilizing an inactive apoenzyme. AngIV binding to the catalytic domain of IRAP has been clearly established in vitro, and the evidence for AngIV binding to an allosteric site in the juxtamembrane region has been questioned. [^{125}I]AngIV binds in an a 1:1 ratio to in the presence and absence of the juxtamembrane region, suggesting that there is only one AngIV binding site but the site is not located in the juxtamembrane region. AngIV binding to the apoenzyme can be measured in vitro, reflecting possibly a different mechanism of binding and stabilization of AngIV when Zn is removed. Taken together, AngIV appears to bind better to the IRAP apoenzyme than the native IRAP. AngIV binding to native AP-N or to the AP-N apoenzyme is poor (Demaegdt et al., 2006), indicating that AP-N as an in vivo receptor for AngIV is poorly supported by data. Thus, redundancy of mechanism of AngIV receptors is possible.

2. Effects on Insulin Regulated Amino Peptidase-Dependent Neuropeptide Potentiation. On the basis of the kinetics of inhibition, AngIV and its analogs have been proposed as in vivo inhibitors of neuropeptide catabolism by IRAP (Ye et al., 2007). AngIV and divalinal display competitive kinetics, indicating that AngIV binding site ligands mediate their peptidase inhibitor effects by binding to the catalytic site of IRAP (Lew et al., 2003). IRAP is known to play a role in the in vivo metabolism of vasopressin and Lys-bradykinin bound to the enzyme (Herbst et al., 1997; Wallis et al., 2007). Substrates identified in vitro for IRAP include vasopressin (AVP), oxytocin, somatostatin, Leu- and Met-enkephalin, and Lys-bradykinin (Herbst et al., 1997; Matsumoto et al., 2001; Lew et al., 2003). These neuropeptides enhance memory consolidation and retrieval (Vauquelin et al., 2002b; Wallis et al., 2007). The physiologic effects of AngIV binding site ligands therefore may result from the inhibition of cleavage of neuropeptides by IRAP altering memory processing (Lew et al., 2003). Accumulation of neuropeptides when AngIV binds to IRAP has been suggested as a mechanism for cognitive enhancement. In a recent study it

was shown that the increase of extracellular dopamine concentration and the anticonvulsant effect of AngIV could be inhibited by a somatostatin receptor antagonist administered in the rat striatum (Li et al., 2009a). Accumulation of somatostatin to mediate these effects may be one possible mechanism for the observed phenomenon, although somatostatin cleavage by IRAP was not confirmed. At the moment there is no direct in vivo evidence that supports the IRAP/AngIV binding-mediated neuropeptide potentiation hypothesis.

3. AngIV Effects on IRAP Trafficking. Although AngIV and its analogs are not cleaved by IRAP, they competitively inhibit the in vitro catalytic activity of IRAP. On this basis, it has been suggested that IRAP inhibitor binding may prolong the cell surface localization of IRAP/AngIV binding sites in neurons to potentiate cognitive and memory functions (Heimfeld et al., 1992; Demaegdt et al., 2004a,b). In tissues not directly involved in cognitive functions, insulin and some IRAP substrates peptide hormones have been shown to regulate kinetics of cell-surface trafficking of IRAP. For instance, in vascular endothelial cells in placenta, intracellular vesicle-bound IRAP responds to oxytocin by translocating the IRAP vesicles to the plasma membrane. By catabolizing oxytocin and vasopressin, translocated intracellular vesicle-bound IRAP regulates oxytocin and vasopressin levels in serum during pregnancy and thereby prevents early contractions and premature delivery. Cotranslocation of IRAP and the glucose transporter GLUT4 associated vesicles to the plasma membrane in adipocytes is stimulated by insulin (Demaegdt et al., 2011). The cytosolic N-terminal domain of IRAP binds p115, a protein that mediates transport vesicle fusion and is cotranslocated with IRAP and GLUT4 to the plasma membrane (Hosaka et al., 2005). The intracellular domain of IRAP plays a role in regulating GLUT4 vesicle trafficking through interaction with specific proteins including AS160 (Larance et al., 2005; Peck et al., 2006; Albiston et al., 2007). GLUT4 has a critical role in insulin-stimulated glucose uptake in these cells (Keller et al., 1995; Ross et al., 1996). The subcellular localization of IRAP in neurons demonstrated striking parallels with distribution of IRAP in insulin responsive adipocytes and muscle cells, where the enzyme plays a role in insulin-regulated glucose uptake. Therefore, the function of IRAP in neurons may be similar to that in insulin responsive cells (Fernando et al., 2007). Relevant to cognitive function of IRAP/AngIV binding site in brain, the majority of IRAP was localized in intracellular vesicles in mouse brain neurons (Fernando et al., 2005, 2007). The binding of IRAP inhibitors may prolong the cell surface localization of IRAP/AngIV binding site and GLUT4 in neurons. The resulting increase of glucose uptake may be responsible for IRAP ligands to facilitate memory in hippocampus-dependent memory deficits in models for amnesia (Fernando et al., 2007; Stragier et al., 2008). The IRAP inhibitors enhance cAMP-stimulated or depolarization-evoked glucose uptake mediated by GLUT4 (Fernando et al., 2008). Although both AngIV and LVV-H7 potentiate glucose uptake into cultured hippocampal neurons in culture, these effects were not confirmed in vivo. Extracellular hippocampal glucose levels significantly decreased while the animals explored the maze. When the animals were not in a maze, glucose levels in their hippocampus remained constant. However, the same analysis performed in the plus maze test showed that both AngIV and LVV-H7 did not significantly alter hippocampal glucose levels compared with control. Increased glucose availability for hippocampal neurons as the basis of enhanced memory seems unlikely.

E. Distribution of the of AngIV Binding Sites

Consistent with the functional definition, the [^{125}I]AngIV autoradiographic mapping demonstrated that highest densities of the putative AngIV binding site are localized in brain regions associated with cognition and sensomotor functions (Moeller et al., 1996). The brain distributions of the AngIV binding sites mapped are confirmed for cross-species consistency in rat, guinea pig, rhesus monkey (Miller-Wing et al., 1993; Roberts et al., 1995; Wright et al., 1995; Moeller et al., 1996), and human brain slices (Moeller et al., 1996; Chai et al., 2000). Binding of [^{125}I]AngIV was reported in brain structures associated with motor functions (Moeller et al., 1995, 1996; Wright and Harding, 1995), in the forebrain (Miller-Wing et al., 1993; Moeller et al., 1995, 1996), in the cerebellum (Miller-Wing et al., 1993), and in the primary motor neocortex (Moeller et al., 1996). The brain autonomic nuclei, dorsal motor nucleus of the vagus, nucleus ambiguus, rostral ventral lateral medulla, and paraventricular nucleus of the hypothalamus are sites of high AngIV binding site density (Moeller et al., 1995, 1996). Lower density of AngIV binding sites exists in sensory structures and primary sensory neocortex (Miller-Wing et al., 1993; Moeller et al., 1995, 1996). It is also well known that AngIV is generated from AngII or AngIII and present in the circulation (Semple et al., 1976; Abhold and Harding, 1988). Independent studies demonstrated that a hemoglobin β-chain fragment isolated from sheep brain with a sequence, Leu-Val-Val-hemorphin-7 (LVV-H7) is also capable of binding and activating the in vivo physiologic effects attributed to activation of putative AngIV binding sites (Moeller et al., 1997). The distribution of ^{125}I-LVV-H7 binding sites in brain slices was identical to those for [^{125}I]AngIV, suggesting that it is an endogenous AngIV binding site ligand (Moeller et al., 1997, 1998; Lee et al., 2001, 2003).

Distribution of [^{125}I]AngIV binding sites have been documented in different species for peripheral tissues, including bovine adrenal cortex ($B_{max} \approx 4$ pmol/mg) (Harding et al., 1994); monkey and rat kidney ($B_{max} \approx$ 1.0 pmol/mg) (Harding et al., 1994), rat, guinea pig, and rabbit hearts ($B_{max} \approx 0.3$-0.8 pmol/mg); and guinea pig

and bovine vascular smooth muscle ($B_{max} \approx 1.0$ pmol/mg). Mapping the binding of AngIV in rat kidney demonstrated high density of AngIV binding site in the outer stripe of the medulla, the glomeruli, and the medullary core. Surprisingly the AngIV binding map is distinctly different from AngIV binding site distribution map in kidney regions (Harding et al., 1994; Coleman et al., 1998). More recently AngIV binding site binding sites were described in mouse macrophages. The observations that the transcription of the IRAP gene in these cells was upregulated in proinflammatory M1-activated macrophages and the cell surface AngIV binding site binding is modulated upon exposure to IFN-γ, LPS, or exogenous particles suggest a possible function of AngIV binding sites in these cells (Nikolaou et al., 2014).

Although the distribution of AngIV is not known in central and peripheral tissue location of AngIV binding sites, the ubiquitous presence of amino peptidases, AP-A and AP-N, can locally produce AngIV from AngII. Both these enzymes have been localized to the plasma membrane of pericytes, suggesting that AngIV can be produced in the extracellular space surrounding microvessels in the brain (Healy and Wilk, 1993; Kunz et al., 1994). Several reports indicate that, within neurons, nearly 80% of AngII is converted to AngIV (Stragier et al., 2008). Exogenous administration of AngIV has been shown to increase cerebral microcirculation (Naveri et al., 1994; Kramar et al., 1997; Kramar et al., 1998; Lanckmans et al., 2007a,b). In terms of function, intracranially administered AngIV and its functional analogs facilitate memory in rodent behavior models (Braszko et al., 1988; Wright et al., 1993; Tchekalarova et al., 2001; Lee et al., 2003). AngIV also facilitates reversal of memory shortfalls produced by scopolamine, mecamylamine, abusive alcohol dose, ischemia, as well as by disruption of the perforant path in the hippocampus (Borawska et al., 1989; Wright et al., 1996; Pederson et al., 1998; Albiston et al., 2004a; Olson et al., 2004). AngIV enhances neuronal long-term potentiation in vitro and in vivo (Kramar et al., 2001; Wayner et al., 2001) as well as attenuates PTZ-induced seizures (Tchekalarova et al., 2001; Stragier et al., 2006) and protects against cerebral ischemia (Faure et al., 2006a,b, 2008). It is reported that in peripheral tissue AngIV administration increases renal blood flow that was blocked by antagonist divalinal-AngIV (Coleman et al., 1998; Hamilton et al., 2001). This effect was also accompanied with an increased urinary sodium excretion (Hamilton et al., 2001). Contrasting results have been reported indicating that AngIV decreased renal blood flow (Gardiner et al., 1993; Fitzgerald et al., 1999; Yang et al., 2008) and increased blood pressure (Yang et al., 2008). The latter effects were prevented by AT_1 receptor antagonists.

F. Insulin Regulated Amino Peptidase Gene Knockout Mice

1. Evaluation of AngIV-dependent Functions. Considering the wide-ranging effects mediated by IRAP and its inhibitors, understanding the genetic basis of IRAP functions is critical. In normal and memory-compromised rodent models, enhancement of memory upon intracerebroventricular delivery of AngIV and its analog peptides is the defining pharmacological property, which is thought to be mediated solely by the IRAP/AngIV binding site (Albiston et al., 2004b; Lee et al., 2004). Biochemically, these ligands show high-affinity binding to the IRAP catalytic site. Therefore, the inhibition of IRAP activity or genetic loss of expression would be expected to improve memory.

Albiston et al. (2010) evaluated effects of IRAP gene knockout on AngIV binding and the behavioral phenotype in mice. In the IRAP-null mouse, the complete loss of IRAP expression in muscle, fat, and brain including hippocampus was accompanied with loss of binding sites for the radioligand [^{125}I]Nle1-AngIV. However, the IRAP-null genotype did not significantly affect the glucose transporter GLUT4 levels in the hippocampus and cortex. There was no detectable difference in the response to depolarization-evoked glucose uptake in the hippocampal slices of the IRAP-null mice compared with their wild-type littermates. Comprehensive neurologic testing of adult IRAP-null mice confirmed that the sensory perception, their motor reflexes, and vestibular function were normal. The performance of young knockout mice in memory and behavioral testing paradigms was similar to the wild-type mice. In the IRAP-null mice, the sensory, locomotor, and anxiety responses were not altered at 3–4 months of age. Furthermore, the IRAP knockout mice demonstrated improved cognitive and spatial memory when tested at 6 and 12 months of age on the Y maze task. A limited impact of IRAP-null genotype in enhancing memory was unexpected, questioning the basis of linking IRAP-inhibition to memory and cognitive enhancement.

The unexpected phenotype could be the result of developmental alteration due to germ-line deletion of the IRAP gene causing susceptibility to age-related cognitive decline. Several questions regarding IRAP-null mice remain unanswered. For instance, whether cognitive enhancement from AngIV and analogs of AngIV is completely abolished in the IRAP-null mice. The authors have demonstrated that IRAP-null mice show complete loss of AngIV binding using tissue imaging methods; they did not examine whether central infusion of AngIV or other IRAP inhibitors had any functional effect on improving performance in a range of memory paradigms or whether a compensatory mechanism is activated as a stand-in for the loss of IRAP in specific neurons. Wallis et al. (2007) reported increased levels of plasma AVP in the IRAP-null mouse,

suggesting that IRAP may play a role in regulating levels of vasopressin. However, it was difficult to reconcile IRAP−/− phenotype with increased AVP and rather extensive literature on AVP effects on different brain regions. Does IRAP play a role in only disease paradigms while an unknown molecule functions as the mediator of physiologic effects of AngIV? Loyens et al. (2011) attempted to examine this question in a recent report, demonstrating that IRAP-null mice show decreased susceptibility to pentylenetetrazol (PTZ)-induced seizures. They observed that thresholds for myoclonic twitch and generalized clonic seizures significantly increased in IRAP(−/−) mice compared with their IRAP(+/+) littermates when PTZ is injected demonstrating that that IRAP(−/−) mice are less sensitive to the development of PTZ-induced seizures. The authors concluded that IRAP is involved in generalized seizure generation in normal mice. Pham et al. (2012) demonstrated that deletion of the IRAP gene protects the brain from ischemic damage analogous to the effect of the IRAP inhibitor AngIV. Transient middle cerebral artery occlusion in mice produces stroke-induced neurologic deficits 24 hours after reperfusion in mice. The infarct volumes measured were significantly reduced in the IRAP knockout mice compared with wild-type littermates, with corresponding improvement in neurologic performance. Cerebral blood flow was increased in the IRAP knockout animals.

2. Nuances for Defining Receptor(s) for AngIV. More than a decade of research focused on delineating the IRAP/AngIV binding site as the sole AngIV binding site has exposed some pharmacological, signaling, and genetic criteria not satisfactorily met by this protein. Other binding proteins have been proposed as alternative to the concept of IRAP/AngIV binding site for AngIV (De Bundel et al., 2008; Stragier et al., 2008; Wright et al., 2008).

AP-N, another transmembrane peptidase structurally related to IRAP/AngIV binding site was suggested as an additional candidate AngIV receptor, because this protein can activate IP3-dependent intracellular calcium and MAPK signaling (Navarrete Santos et al., 2000b,c). However, evidence demonstrating dimerization of AP-N and signaling in response to AngIV binding is lacking. More importantly, the role of this protein in AngIV-mediated cognitive enhancement is currently not established.

The ability of AngIV analogs to alter cognitive function and augment neurite outgrowth in vitro led to the hypothesis that the c-Met tyrosine kinase receptor for the hepatocyte growth factor (HGF) is a candidate AngIV binding site (Wright et al., 2008). Sequence-based prediction identified the possibility for AngIV and its analogs to disrupt HGF interaction with c-Met. As predicted, in picomolar concentration Norleual-AngIV was found to displace HGF binding to c-Met and antagonize signaling activity of HGF through c-Met in vitro. Norleual-AngIV blocks HGF-induced proliferation, invasion, cell scattering, and wound closure in vitro; inhibits ex vivo angiogenesis; and attenuates melanoma lung colonization in vivo. Some of the HGF/c-Met functions partially overlap with classic functions attributed to the AngIV binding site, including facilitated memory consolidation and hippocampal LTP and calcium signaling. These studies suggest that the c-Met is a cellular receptor candidate for mediating biologic effects of picomolar concentrations of AngIV analogs. These authors suggested MSP/Ron receptor system as an additional potential target of AngIV analogs that warrants investigation (Wagh et al., 2008). A number of questions regarding c-Met as a potential AngIV binding site remain unexplored. For instance, can AngIV specifically activate and AngIV-antagonists inhibit the c-Met receptor signaling in vivo to reproduce functions attributed to classic AngIV/AngIV binding site system? Does the distribution of c-Met receptor in brain and its levels within cognitive centers correlate with the classic mapped AngIV binding sites? Does LVV-H7 and the other IRAP-selective ligands discussed above bind to the c-Met receptor? Does recombinant c-Met show high-affinity binding to AngIV and LVV-H7?

3. AngIV Binding Site Conclusion. The state of IRAP/AngIV binding site protein as the sole receptor for mediating in vivo functions of AngIV will have to be revisited in future. Multiple lines of evidences suggest that IRAP/AngIV binding site protein is a high-affinity AngIV binding receptor that mediates some of the neuroprotective and cognitive effects of AngIV, although the exact signaling mechanisms mediating these effects have not been clearly established. The IRAP/AngIV binding site hypothesis has stimulated development of rather potent and specific inhibitors of its enzymatic function, which have been shown to be cognitive enhancers in vivo. At present existing data indicate that IRAP/AngIV binding site protein does not discriminate between agonist and antagonist ligands and does not directly account for transmembrane signaling. Better characterization of IRAP/AngIV binding site inhibitors using a broad range of assays may unravel intricacies of AngIV functions in vivo. Future developments in this field are likely to uncover additional AngIV receptors and mechanisms of AngIV action in vivo. Such receptors will have to address several aspects of AngIV physiology, for example: i) in vivo efficacy at picomolar and subnanomolar concentrations at which AngIV and its analogs were found to affect changes in physiologic function; ii) the rapid onset of the physiologic effects of AngIV in blood flow and intracellular calcium signaling or neuronal potentiation experiments; iii) pharmacological effects of the "agonists" and "antagonists" on the receptor-specific effects on downstream signaling; and iv) global genetic deletion of receptor abolishes pharmacological and physiologic effects of AngIV and increases

susceptibility to neurodegeneration or decline of cognitive functions. This is critical because the AngIV/AngIV binding site system is implicated in cognitive, memory, and cerebroprotective functions, as well as disease conditions like Alzheimer's, seizures, and Parkinson's disease. The lack of AngIV binding site agonists and antagonists penetrating blood-brain barrier has limited our understanding of importance in the etiology and treatment of memory dysfunctions associated with dementia and degenerative diseases.

VI. Receptor Conundrum in the Renin-Angiotensin System

Based on the multiple criteria used to recommend the pairing of an orphan receptor with its cognate ligand, MAS was included among the "orphan" GPCRs, for which the status of endogenous ligand(s) remains unclear (Davenport et al., 2013). The status of MAS is unchanged in the current update to IUPHAR/BPS Guide to Pharmacology. MAS is a candidate receptor for endogenously produced RAS peptide hormone Ang(1-7) (Tirupula et al., 2014, http://www.guidetopharmacology.org/GRAC/ObjectDisplayForward?objectId=150.).

MAS was initially discovered in 1986 as an oncogene that transformed NIH3T3 cells and induced tumors in nude mice (Young et al., 1986). The amino acid sequence of MAS bears fingerprint of GPCRs, and tumorigenic activity of overexpressed receptor was confirmed later (Janssen et al., 1988; van 't Veer et al., 1993). However, the genomic *Mas* as an oncogene is disputed, because this gene is not amplified in tumors. Overexpression of MAS, however, can transform cells. MAS was originally proposed as a functional receptor for AngII that also mediated response to angiotensins I and III in *Xenopus* oocytes (Jackson et al., 1988). In transfected cells, MAS mediated intracellular calcium mobilization and DNA synthesis responses to AngII and III (Jackson et al., 1988; Jackson and Hanley, 1989; McGillis et al., 1989; Dean and Boynton, 1990). However, the status of MAS as AngII receptor was disproved. The cell lines where AngII and MAS interactions were characterized expressed endogenous AT_1 receptor (Ambroz et al., 1991; Monnot et al., 1991; Murphy et al., 1991; Sasaki et al., 1991), thus ending the controversy of AngII activating MAS (Sasamura et al., 1992).

A. Pairing MAS Receptor with Ang (1-7)

Current peer review literature supports pairing of MAS with multiple endogenous peptides with preference toward studies involving Ang(1-7). Originally discovered as a GPCR with transforming activity (1986) and the founding member of the Mas-related G-protein–coupled receptor subfamily of neurohormone receptors (2001), MAS remained orphan until the neuropeptide FF was shown to activate G-protein signaling through this receptor (see Fig. 9). Two years later, MAS-transfected cells were shown to produce arachidonic acid response to Ang(1-7) (Santos et al., 2003b; Gembardt et al., 2008; Zimpelmann and Burns, 2009). The angiotensin-converting enzyme 2 metabolizes AngI and AngII to Ang(1-7) (IUPHAR-DB ID no. 582), which functions as a vasodilator and antiproliferative agent. Some of the actions of Ang(1-7) appear to be mediated via MAS in vivo (Santos et al., 2003b). Deletion of the *Mas* gene in mice abolishes the binding of Ang(1-7) in mouse kidney as well as abolishes the antidiuretic action of the peptide after an acute water load (Santos et al., 2003b). Many in vivo effects attributed to Ang(1-7) are diminished or absent in *Mas*-null mouse tissues and organs, providing support for the proposed ligand receptor pairing (Walther et al., 1998; da Costa Goncalves et al., 2007; Fraga-Silva et al., 2008; Santos et al., 2008; Pinheiro et al., 2009; Mario et al., 2012). An evolutionary account of MAS and related GPCRs is presented in an extensive review recently by Bader et al. (2014). Hence, further input from the scientific community is needed to validate MAS as the cognate receptor for Ang(1-7).

Alamandine is a relatively new heptapeptide that contains an Ala replacing Asp at position 1 of Ang(1-7). This peptide can be generated by either decarboxylation of the Asp residue of angiotensin-(1-7) or by catalytic action of angiotensin-converting enzyme-2 on decarboxylated AngII (also called angiotensin A) (Lautner et al., 2013). Two primary journal articles on alamandine, since its discovery, have paired this ligand with Mas-related G-protein–coupled receptor D; however, neither strong pharmacological nor direct radioligand binding data exist (Habiyakare et al., 2014; Wilson et al., 2015). Identification of alamandine and its putative receptor has attracted attention as a novel mediator of physiologic and pathophysiological actions of the RAS and may help to develop new therapeutic strategies for treating human cardiovascular diseases.

Conventional G-protein signaling by MAS, reported to be stimulated by the physiologic ligand neuropeptide FF (Dong et al., 2001), was recently confirmed (Tirupula et al., 2014). Reports pairing MAS with several other physiologic and synthetic peptides exist, such as with angiotensin III, angiotensin IV, angioprotectin P33/CGEN-857, P61/CGEN-856, and MBP-7 (Bikkavilli et al., 2006; Gembardt et al., 2008; Shemesh et al., 2008; Savergnini et al., 2010; Jankowski et al., 2011). Consequently, these other physiologic peptides reported as endogenous ligands for MAS remain incompletely characterized. Few reports indicate that G-proteins are activated by MAS in response to few physiologic peptides and "surrogate" nonpeptide ligands in transfected cells but not in response to Ang(1-7) (Bikkavilli et al., 2006; Canals et al., 2006; Shemesh et al., 2008; Tirupula et al., 2014; Zhang et al., 2012). MAS shares significant similarity with Mas-related G-protein–coupled receptors or paralogs, which are predominantly expressed in

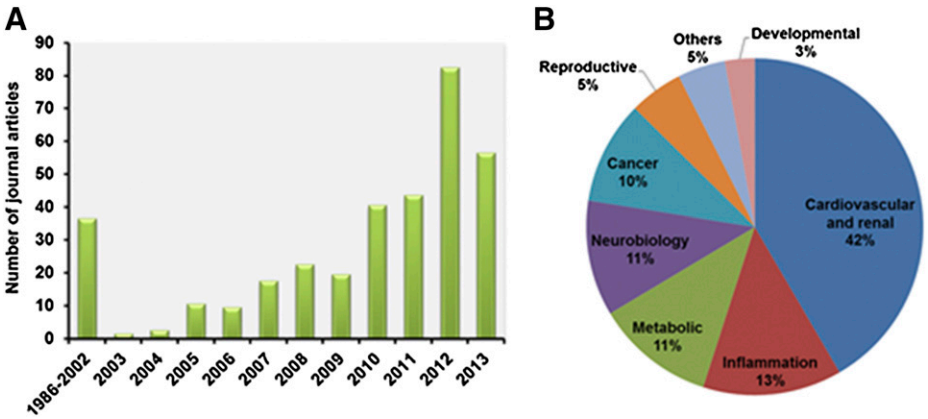

Fig. 9. Literature search and analysis of primary journal articles on MAS. The journal articles published on MAS were automatically retrieved from PubMed with the search term "(((((MAS[Title/Abstract]) AND angiotensin OR MAS1[Title/Abstract]) AND angiotensin[Title/Abstract]) NOT meconium aspiration syndrome[Title/Abstract]) NOT medication adherence scale[Title/Abstract])". The results were manually curated to add or remove relevant or irrelevant references, respectively, and the primary journal articles were separated from the reviews, interviews, lectures, or commentary articles. The final curated list had a total of 337 primary journal articles until the end of year 2013. These articles are further analyzed to illustrate (A) number of publications per year as a bar graph and (B) percentage of publications per functional category as a pie chart. The functional categorization was done by searching for key words in the abstract and the title representing a given category. Please note that in the functional categorization, some articles are present in multiple functional categories and therefore 100% represents a higher total of articles (524) instead of 337. The figure helps to illustrate the general point that the number of publications on MAS have dramatically increased post-2003 and that MAS literature is primarily representative of the cardiovascular and renal function category. Python and Biopython scripts were used to aid in the literature search and analysis.

neurons and immune cells (Young et al., 1986; Dong et al., 2001; Bender et al., 2002). MAS might be expected to respond to physiologic ligands, which are typically neuropeptides and pro- or anti-inflammatory ligands (Dong et al., 2001; Lembo et al., 2002; Choi and Lahn, 2003; Tatemoto et al., 2006; Liu et al., 2009; Kashem et al., 2011; Subramanian et al., 2011; Han et al., 2013). Therefore, the role of MAS as the receptor for only Ang(1-7) qualifying it as an integral constituent of the RAS is unclear. MAS appears to be a GPCR with high constitutive activity and may not have a unique cognate endogenous ligand (Canals et al., 2006; Zhang et al., 2012; Tirupula et al., 2014). Many physiologic modulators of MAS activity may exist; important among them may include peptides such as Ang(1-7) and neuropeptide FF. Only when a clear understanding of the physiology, pharmacology, and pathology for all ligands of MAS has begun to emerge, can the status of MAS be assigned with greater certainty. The current objective is to stimulate research into confirming multiple ligands pairing with MAS in physiologic regulations. A brief perspective on MAS given below summarizes its potential relationship to RAS and pathophysiological regulation in vivo.

B. Pharmacology

Using *Mas*-knockout mice in studies, Santos et al. (2003b) first proposed that Ang(1-7) is the physiologic ligand for MAS, a discovery that has transformed research on MAS (see Figs. 9 and 10). The basis for initial proposal of Ang(1-7) as an endogenous MAS ligand is the observed loss of Ang(1-7) tissue effects in the MAS-null mice. Proposed pairing of Ang(1-7) with MAS was further confirmed in receptor binding and functional studies in MAS-transfected cells. Specific [^{125}I]Ang(1-7) binding to kidney sections was reduced in MAS-null mice (Santos et al., 2003b; Pinheiro et al., 2004) as well as in MAS-GFP transfected cells (Gironacci et al., 2011). The Ang(1-7) antagonist A-779 competed with an IC_{50} close to the K_d of ^{125}I-Ang(1-7) in Mas-transfected cells (Santos et al., 2003b; Gironacci et al., 2011). Binding specificity to MAS was reported by using fluorescently labeled Ang(1-7) in CHO cells (Pinheiro et al., 2004; Savergnini et al., 2010; Jankowski et al., 2011), platelets (Fraga-Silva et al., 2008), and Leydig cells (Leal et al., 2009). Specific binding of fluorescently labeled Ang(1-7) was absent in MAS-null mice and the binding is blocked by A-779 and AVE0991, a nonpeptide analog of Ang(1-7) (Wiemer et al., 2002). However, in most of the published reports on MAS, standard pharmacological binding experiments are absent, presumably due to technical problems. Overall, radiolabeled or fluorescently labeled Ang(1-7) data in most reports intended to confirm a direct interaction between Ang(1-7) and MAS are of poor pharmacological rigor. This is a serious setback in the deorphanizing endeavor.

C. Signaling

Ang(1-7) treatment causes concentration-dependent release of arachidonic acid by stimulating mitogen-activated protein kinase p38 in human mesangial cells and MAS-transfected CHO and COS cells (Santos et al., 2003b; Gembardt et al., 2008; Zimpelmann and Burns, 2009). Ligand treatment with Ang(1-7) or AVE0991 in MAS-transfected cells couples calcium-independent activation of nitric oxide synthase to the phosphatidylinositol 3-kinase/protein kinase B, AKT pathway (Sampaio

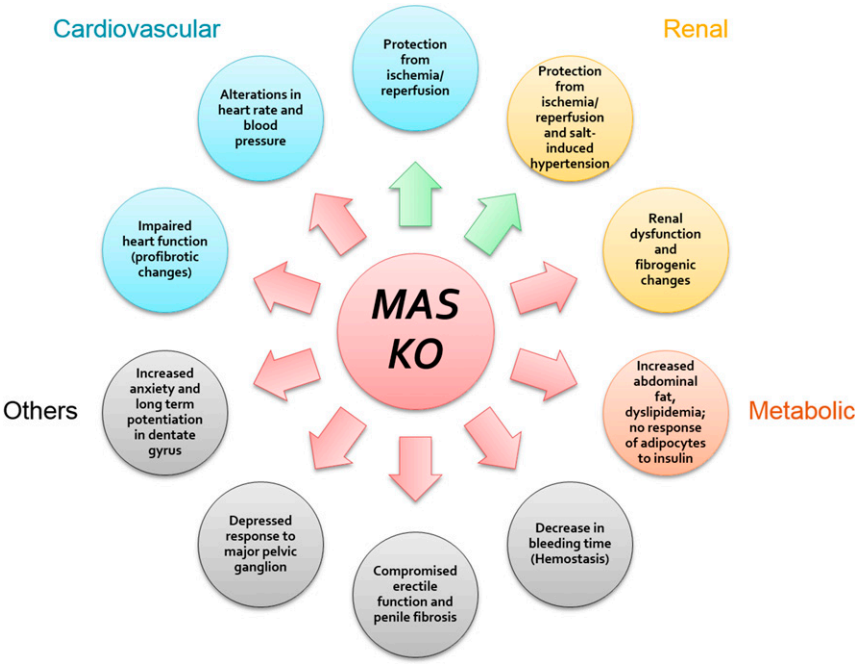

Fig. 10. Summary of phenotypes reported in MAS knockout mice. The figure summarizes multiple phenotypes that are reported in literature in mice lacking the MAS gene. Both damaging (red arrows) and protecting (green) arrows are observed in knockout mice. See main text for references.

et al., 2007b; Lopez Verrilli et al., 2012; Savergnini et al., 2013; Than et al., 2013), or activation of phospholipase A2 (Santos et al., 2003b). Ang(1-7) altered the phosphorylation of MAP kinases (Sampaio et al., 2007a; Zimpelmann and Burns, 2009; Liu et al., 2012; Verano-Braga et al., 2012). Thus, Ang(1-7) action through MAS is proposed to be production of arachidonic acid and activation of nitric oxide synthase, which may not involve cAMP, IP3, and calcium signaling.

Although MAS is a GPCR, there is no evidence for Ang(1-7)-mediated conventional G-protein signaling as measured by the levels of second messenger molecules like calcium, IP3, and cAMP in MAS-transfected cells (Bikkavilli et al., 2006; Shemesh et al., 2008; Zhang et al., 2012; Tirupula et al., 2014). This observation is, however, disputed by reports in kidney, where Ang(1-7) treatment is reported to increased cAMP levels and protein kinase A activation, suggesting $G\alpha_s$ activation by MAS. These signals could be inhibited using A-779 (Magaldi et al., 2003; Liu et al., 2012). MAS was shown to constitutively couple to $G\alpha_i$, $G\alpha_q$, and $G\alpha_{12/13}$ proteins (Zohn et al., 1998; Whitehead et al., 2001; Booden et al., 2002; Chen and Ikeda, 2004; Singh et al., 2010a; Tirupula et al., 2014). Adenovirus-mediated overexpression of MAS in rat neonatal cardiomyocytes caused IP3 accumulation and myocyte hypertrophy through $G\alpha_q$-mediated signaling (Zhang et al., 2012). Constitutive activity of the MAS in overexpressing cells activates G-protein signaling pathways (Dias-Peixoto et al., 2008; Shemesh et al., 2008; Gomes et al., 2012; Zhang et al., 2012; Tirupula et al., 2014). Similar to Ang(1-7) the analogs A-779 and AVE 0991 fail to activate G-protein signaling through MAS (Fontes et al., 1994; Wiemer et al., 2002; Kluskens et al., 2009). Thus, the current state of Ang(1-7) interaction with MAS with regards to G-protein activation is controversial.

Conventional G-protein signaling by MAS was reported upon stimulation with another physiologic ligand, neuropeptide FF (Dong et al., 2001). Few other reports indicated that the G-protein activation by MAS was both constitutive and in response to some peptide and synthetic ligands (Bikkavilli et al., 2006; Canals et al., 2006; Shemesh et al., 2008; Zhang et al., 2012; Tirupula et al., 2014). In these studies, disparate ligands such as MBP7 (Bikkavilli et al., 2006), the angiotensin metabolite peptides AngIII and AngIV (Gembardt et al., 2008), novel nonpeptide ligands AR234960 (1-[[(4*S*)-4-(3-fluorophenyl)-1-(2-methoxy-4-nitrophenyl)sulfonylpyrrolidin-3-yl] methyl]-4-pyridin-2-ylpiperazine), AR244555 (1′-but-3-enyl-5-chlorospiro[2*H*-indole-3,4′-piperidine]-1-yl)-(2,6-difluorophenyl)methanone), and AR305352 (Zhang et al., 2012), and CGEN-856/P61 (Peptide sequence: FLGY-CIYLNRKRRGDPAFKRRLRD) (Shemesh et al., 2008; Savergnini et al., 2010) were found to modulate MAS-mediated G-protein signaling pathways at relatively high concentrations of each peptide. Nonpeptide ligands with both agonist and inverse agonist properties and high selectivity for MAS are now known. The agonist AR234960 was shown to activate G-protein signaling and the inverse agonist AR244555 selectively inhibited the agonist response as well as the constitutive activity of MAS (Zhang et al., 2012; Tirupula et al., 2014). Remarkably, CGEN-856S is reported to activate calcium and AKT signals (Shemesh et al., 2008; Savergnini et al., 2013). Thus, atypical signaling response suggests functional selectivity of MAS, which allows

interaction with different ligands to activate different signaling pathways, a phenomenon also called biased signaling (Tirupula et al., 2014).

D. Other Receptors for the Proposed Ligands

Other receptors such as the AT_2 receptor can bind Ang(1-7) (De Souza et al., 2004; Walters et al., 2005). Similarly, NPFF is known to activate distinct GPCRs, NPFFR1 and 2. Thus, it is not clear whether the interaction between these ligands and MAS is mutually exclusive.

E. Tissue Distribution of MAS

The pattern of tissue distribution of MAS is broadly conserved in mammals. The highest expression of MAS is in brain and testis, but in other organs low level expression is detected (Metzger et al., 1995; Alenina et al., 2002). *Mas* gene is expressed in the hippocampus, cerebral cortex, the dentate gyrus, the olfactory tubercle, and the olfactory bulb in rodent brain (Young et al., 1988; Bunnemann et al., 1990); particularly interesting to note is that MAS expression is observed in regions of brain important for cardiovascular regulation (Becker et al., 2007). *Mas* gene expression was also discovered in a wide variety of rodent tissues (Villar and Pedersen, 1994; Metzger et al., 1995; Alenina et al., 2008). The MAS seen in the endothelial cells in blood vessels of different organs including heart is very low (Kumar et al., 1996; Alenina et al., 2008). MAS expression in the rat brain starts at postnatal day 1. In adult rats, MAS level is transiently up regulated after episodes of seizure in the hippocampus (Martin et al., 1992; Martin and Hockfield, 1993). MAS expression starts after birth in testis and its levels increase during puberty (Metzger et al., 1995; Alenina et al., 2002). MAS was proposed to be a component of brain RAS in 1992, based on colocalization mapping of MAS, AngII, and AngII receptors with angiotensinogen, renin, angiotensin converting enzyme, and angiotensin fragments (Bunnemann et al., 1992).

F. Mas Gene-Knockout in Mice.

Availability of *Mas* knockout mice since 1998 provided unique opportunity to study the involvement of MAS in behavioral, cardiovascular, renal processes, and several metabolic physiologies (Walther et al., 1998). Multiple phenotypes are reported in literature for *Mas* knockout mice, both damaging and protective effects on organs and tissue as detailed in Fig. 10.

Mas knockout mice show increased anxiety and altered variability of heart rate and blood pressure in a sex-specific manner (Walther et al., 1998, 2000a,b). The early *Mas* knockout mice were in a mixed genetic background (129×C57BL/6) in which variability in pathophysiological consequences was observed. Significant alterations in cardiovascular and metabolic physiologies were evident only in knockout mice with pure genetic backgrounds like C57BL/6 and FVB/N (Xu et al., 2007; Santos et al., 2008). *Mas* knockout mice harbor impaired cardiac function accompanied with profibrotic expression of extracellular matrix proteins in the hearts (Santos et al., 2006; Gava et al., 2012). Renal dysfunctions associated with fibrotic changes were also reported (Pinheiro et al., 2009). The *Mas* knockout animals have increased expression of the proinflammatory cytokine, transforming growth factor-β, type 1, and increased expression of profibrotic and proinflammatory molecules such as fibronectin, collagen, α-smooth muscle actin, vimentin, connective tissue growth factor (CTGF), and tumor necrosis factor-α (Santos et al., 2008). Overexpression of MAS inhibited the expression of the proinflammatory genes. MAS-deficient mice have increased abdominal fat mass, dyslipidemia, increased levels of insulin and leptin, and altered response of adipocytes to insulin (Santos et al., 2008; Mario et al., 2012). *Mas* knockout offers protection from salt-induced hypertension (Heringer-Walther et al., 2012) and from ischemia/reperfusion injury in both kidney and heart (Castro et al., 2006; Esteban et al., 2009; Zhang et al., 2012). In addition the *Mas* knockout, mice are also reported to have changes in hemostasis (Fraga-Silva et al., 2008) and penile fibrosis (da Costa Goncalves et al., 2007). A common mechanism behind the *Mas* knockout mice, exhibiting altered heart rate and blood pressure, increased vascular resistance, and decrease blood flow, is thought to be endothelial dysfunction due to imbalances in nitric oxide (NO) and reactive oxygen species (Castro et al., 2005; da Costa Goncalves et al., 2007; Peiro et al., 2007; Rabelo et al., 2008; Xu et al., 2008; Rakušan et al., 2010; Botelho-Santos et al., 2012).

G. Pathophysiological Evidence of MAS Receptor Interaction with Ang(1-7).

The vasodilation, antidiuresis, and antithrombosis responses to Ang(1-7) are lost in the *Mas* knockout mice (Santos et al., 2003b; Fraga-Silva et al., 2008). Validation of the cardiovascular actions of Ang(1-7) in the MAS-deficient mice take advantage of agonists AVE 0991 (Wiemer et al., 2002) and CGEN-856S (Shemesh et al., 2008) and the antagonists A-779 (Santos et al., 1994, 2003b) and D-Pro7 Ang(1-7) (Santos et al., 2003a). Based on the important functional evidences obtained in *Mas* knockout mice and the anti-inflammatory and antifibrotic effects of MAS in wild-type mice, MAS was proposed to be an important component of the RAS. Possibilities to enhance in vivo protective functions through Ang(1-7)-mediated activation of MAS make it an enticing drug target (Steckelings et al., 2011b). For example, inhibition of MAS function is shown to offer protection from ischemia/reperfusion injury in both mouse kidney and heart, making it a target receptor in the RAS for drug development (Castro et al., 2006; Esteban et al., 2009; Zhang et al., 2012). However, the physiologic effects of Ang(1-7) treatment combined with

MAS deficiency in mice appear to be very complicated. For example, multifaceted effects of Ang(1-7) in vasculature may involve MAS interaction with AT_1 receptor and AT_2 receptor (Castro et al., 2005). MAS was shown to function as a physiologic antagonist of AT_1 receptor by altering AngII response in the mice (Von Bohlen und Halbach et al., 2000; Kostenis et al., 2005; Rakušan et al., 2010). MAS-AT_1 receptor heterooligomerization resulted in the altered trafficking of AT_1 receptor in transfected cells (Kostenis et al., 2005; Canals et al., 2006; Santos et al., 2007). Thus, the evidence for MAS interacting with AT_1 receptor and AT_2 receptor may be a mechanism for MAS in RAS. Literature regarding Ang (1-7) interaction with MAS in various organs is summarized below, and for a detailed literature compilation we refer to recent review by Bader et al. (2014).

1. Heart. Picomolar concentrations of Ang(1-7) were reported to cause a significant vasodilator effect in isolated rat hearts (Souza et al., 2013). Acute stimulation with Ang(1-7) in cardiomyocytes has no evident effect on calcium transients but stimulates release of NO (Dias-Peixoto et al., 2008; Costa et al., 2010; Gomes et al., 2010). However, an increased uptake and SERCA2 expression in transgenic rats overexpressing Ang(1-7) was reported by Gomes et al. (2012). *Mas* knockout alters expression of calcium handling proteins and decreased contractility of heart in mice (Santos et al., 2006; Botelho-Santos et al., 2012; Gava et al., 2012; Gomes et al., 2012). The cardiomyocytes in *Mas* knockout mice increase caveolin 3 expression and decrease HSP90, which reduces eNOS activity (Wu, 2002; Takahashi and Mendelsohn, 2003; Dias-Peixoto et al., 2008).

MAS RNA expression is reported in cardiomyocytes, cardiac fibroblasts, and the sinoatrial node cells (Ferreira et al., 2011). Ang(1-7) inhibits agonist-stimulated hypertrophy in culture cardiomyocytes (Gallagher et al., 2008; Gomes et al., 2010; Flores-Munoz et al., 2012), which can be blunted by siRNA-mediated knock down of MAS (Tallant et al., 2005). In transgenic rats, elevation of circulating Ang(1-7) causes attenuated cardiac fibrosis in response to isoproterenol (Santos et al., 2004b). In support of this finding, many investigators have evaluated the antiremodeling effects of Ang(1-7) and other MAS agonists, AVE 0991 and CGEN-856S (He et al., 2004; Iwata et al., 2005; Grobe and Katovich, 2006; Iusuf et al., 2008; Mercure et al., 2008; Giani et al., 2010; Gomes et al., 2010; Durik et al., 2012; Patel et al., 2012). Acute blockade of MAS with A-779 produces a deterioration of isolated mouse heart functions (Castro et al., 2005). Thus, independent studies support functional pairing of MAS and Ang(1-7) in myocardium; however, efficacy of this system needs rigorous proof.

2. Kidney. Although MAS expression could not be detected in the human kidney (Shalamanova et al., 2010), MAS transcripts have been detected in the kidney of rodents (Santos et al., 2003b; Pinheiro et al., 2004; da Silveira et al., 2010). *Mas* knockout mice lack the antidiuretic action of Ang(1-7) (Santos and Baracho, 1992). Both the anti- and proinflammatory effects of Ang(1-7) were reported in kidney of *Mas* knockout mice (Esteban et al., 2009; Singh et al., 2010b; Moon et al., 2011; Bernardi et al., 2012; Giani et al., 2012; Harris, 2012; Chou et al., 2013; Santos et al., 2013).

3. Vasculature. Studies performed in MAS models of gain- or loss-of-function show that systemic and local hemodynamics are chronically affected. In humans and other species, the endothelial layer of blood vessels produce Ang(1-7) (Santos and Baracho, 1992) and the presence of MAS in endothelial cells and VSMC is conserved, suggesting localized functional interaction (Santos et al., 2000; da Costa Goncalves et al., 2007; Sampaio et al., 2007b; Xu et al., 2008). *Mas* knockout increases the vascular resistance in mice in various organs (Botelho-Santos et al., 2012). In the transgenic rats, with increased circulating Ang(1-7), an Ang(1-7)-dependent vasodilatory tone in blood vessels is suggested. Endothelial dysfunction is observed for *Mas* knockout in the FVB/N background, causing blood pressure increases (Xu et al., 2008). However, in C57Bl/6 mice, MAS knockout does not alter blood pressure (Rabelo et al., 2008). Short-term Ang(1-7) infusion in normotensive rats enhanced endothelial function (Faria-Silva et al., 2005), which may be also be conserved in other species (Langeveld et al., 2008; Durand et al., 2010; Stegbauer et al., 2011; Beyer et al., 2013; Jarajapu et al., 2013; Tassone et al., 2013).

4. Brain. Reduction in baroreflex sensitivity and sympathetic activity is observed in *Mas*-null mice (Walther et al., 2000b). In addition Ang(1-7) and MAS provide a protective effect in models of stroke (Mecca et al., 2011; Regenhardt et al., 2013, 2014a,b). MAS is present in select regions of the brain, which provides the structural basis for many effects produced by Ang(1-7) in the brain. For instance, the modulation of sympathetic activity (Silva et al., 1993; Fontes et al., 1994; da Silva et al., 2011; Kar et al., 2011; Li et al., 2013), increase of vagal tone (Guimaraes et al., 2012), and improvement of baroreflex sensitivity (Chaves et al., 2000) are Ang(1-7) responses, and these effects have been shown to be blocked by the MAS antagonist A-779.

5. Reproductive Organs. MAS is expressed in the testis, and changes in its expression are regulated during puberty (Metzger et al., 1995; Alenina et al., 2002). However, fertility is not affected in *Mas* knockout mice (Walther et al., 1998), but an increased risk of fibrosis and erectile dysfunction was reported (da Costa Goncalves et al., 2007). In humans, correlation between MAS expression in seminiferous tubules and male infertility was observed (Reis et al., 2010). MAS gene expression was reported in human ovaries (Reis et al., 2011). Both Ang(1-7) and MAS modulate folliculogenesis, ovulation, and pregnancy (Viana et al., 2011).

Consistent with a regulatory role of MAS in ovary, the MAS antagonist A-779 was shown to prevent breakdown of germinal vesicle and reduced oocyte maturation induced by Ang(1-7) (Honorato-Sampaio et al., 2012). Expression of MAS in uterus and placenta is thought to be relevant to diseases such as preeclampsia (Velloso et al., 2007). Thus, MAS may not be important modulator of male and female fertility but may be relevant in pathogenesis related to fertility.

H. Conclusion

The current literature on MAS lacks convincing evidence for a direct interaction between MAS and Ang(1-7) with a potency that is consistent with a physiologic functions altered by Ang(1-7) and *Mas* knockout. Although independent groups have supported this pairing between MAS and Ang(1-7) in cellular functional and in vivo physiologic studies, caution expressed is based on basic pharmacological and mechanistic experiments, which are relevant and need to be addressed. The status of other possible ligands pairing with MAS and physiologic function for those ligands through MAS are important additional factors. Therefore, Ang(1-7)/MAS pairing remains controversial and more research is required for NC-IUPHAR to make a formal assignment of MAS as the receptor for the RAS hormone Ang(1-7).

VII. Overall Summary

The angiotensin receptor field has seen enormous development in the last 15 years on the structure, pharmacology, signaling, physiology, and disease states related to these receptors. The importance of each receptor in cardiovascular, hemodynamic, neurologic, renal, and endothelial functions, as well as in cell proliferation, survival, matrix-cell interaction, and inflammation, seems very well recognized. Therefore therapeutic agents targeted to these receptors are either in active use in clinical intervention of common diseases or under evaluation for repurposing in many other disorders.

Conclusions drawn earlier that the majority of known cardiovascular effects of AngII are mediated by the AT_1 receptor is firmly established. Precision analysis of various facets of AT_1 receptor mechanism has taken the place in explaining processes such as vasoconstriction, electrolyte-water balance, cellular proliferation and hypertrophy, and migration and extracellular matrix regulation. Precision analysis of function seems to be the new vogue in AT_1 receptor research, resulting in efforts to dissect out a range of questions directed to cell-specific functions, ligand-specific signals, and inhibitor-specific therapeutic applications. Availability of three-dimensional structures for AT_1 receptor bound to anti-hypertensive agents is expected to advance into new areas of AT_1 receptor structure-function. New signaling modalities, such as functional selectivity of AngII analogs for enhancing β-arrestin–biased protective signaling, are now entering Phase II clinical studies for the treatment of acute HF and may do so to support vasculature and kidney function. AT_1 receptor role in transactivation of receptor tyrosine kinases and mediating mechanical stress is gaining attention. Thus, position of AT_1 receptor as a master regulator is well established.

The AT_2 receptor as a functional antagonist of AT_1 receptor has unraveled through studies of genetically altered mouse models, signal transduction analysis, and, most importantly, through development of new generation of ligands targeting this receptor. The literature on the AT_2 receptor is quite extensive, demonstrating unconventional ligand selectivity, agonist-independent signaling, and cGMP/NO signaling by AT_2 receptor. Identification of novel AT_2 receptor–interacting proteins in recent years has rekindled interest in signaling mechanisms of this enigmatic receptor. Development of pharmacological agents targeted to AT_2 receptor could find therapeutic application in protection of kidney, pain management, and stimulation of nerve growth.

The status of AngIV binding site and MAS as angiotensinergic mediators is still evolving. Functional studies clearly demonstrate important roles for these in central nervous system and in renal and cardiovascular regulation. Especially, MAS interaction with Ang(1-7) has gained significant attention. Multiple receptor candidates and multiple endogenous ligands for the AngIV binding site as well as for MAS may be physiologic. However, pharmacological characterization of these physiologic entities do not rise to the level of acceptance as specific angiotensin receptors at this time. Rigorous pharmacology and signaling studies are warranted.

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Authorship Contributions

Wrote or contributed to the writing of the manuscript: Karnik, Unal, Kemp, Tirupula, Eguchi, Vanderheyden, Thomas.

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