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Dimerization of AT_2 and Mas Receptors in Control of Blood Pressure

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Abstract

Purpose of Review—Angiotensin type 2 receptor (AT_2R) and receptor Mas (MasR) are part of the "protective arm" of the renin angiotensin system. Gene and pharmacological manipulation studies reveal that AT_2R and MasR are involved in natriuretic, vasodilatory, and anti-inflammatory responses and in lowering blood pressure in various animal models under normal and pathological conditions such as salt-sensitive hypertension, obesity, and diabetes. The scope of this review is to discuss colocalization and heterodimerization as potential molecular mechanisms of AT_2R- and MasR-mediated functions including antihypertensive activities.

Recent Findings—Accumulating evidences show that AT_2R and MasR are co-localized, make a heterodimer, and are functionally interdependent in producing their physiological responses. Moreover, ang-(1–7) preferably may be an AT_1R-biased agonist while acting as a MasR agonist.

Summary—The physical interactions of AT_2R and MasR appear to be an important mechanism by which these receptors are involved in blood pressure regulation and antihypertensive activity. Whether heteromers of these receptors influence affinity or efficacy of endogenous or synthetic agonists remains a question to be considered.

Keywords

Angiotensin II type 2 receptor; Mas receptor; Angiotensin II type 1 receptor; Dimerization; Functional interdependence; Blood pressure

Introduction

Renin angiotensin system (RAS) is an important hormone system known to regulate volume homeostasis and BP. RAS is comprised of various enzymes, bioactive peptides, and receptors, which produce diverse and opposing cellular and physiological responses. Angiotensin-converting enzyme (ACE) and angiotensin II (ang-II) and its type 1 receptor (AT_1R), collectively termed as "deleterious arm" of RAS, are involved in the pathogenesis of

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hypertension including vasoconstriction and anti-diuresis/anti-natriuresis. Contrarily, ang-II type 2 receptor (AT_2R), ACE2, ang-(1–7), and MasR, collectively termed as "protective arm" of the RAS, have been shown to play role in vasodilatation, promoting diuresis/natriuresis, and lowering BP, thus largely counteracting the effects mediated via the AT_1R. Although the threeRAS receptors, namely AT_1R, AT_2R, and MasR, have been assigned to their specific cellular and physiological responses, evidences have been documented indicating that these receptors affect each other's cellular expression, signaling, and response. For example, the absence of the AT_2R enhances the AT_1R-mediated cellular response and BP [1–4] and an increased expression of the AT_2R attenuates the AT_1R-mediated signaling [5] and BP [6, 7]. Similarly, AT_1R-mediated responses decrease upon activation of the MasR [8]. As it relates to the expression, renal MasR expression is decreased in AT_2R knockout mice [1] and the activation of the AT_2R causes an increase in the kidney MasR expression [9]. Of the proposed mechanisms include physical interaction of AT_2R [10] or MasR [11] with AT_1R and/or post-receptor opposing signaling cross talk. Since the expression of AT_1R, relative to the AT_2R and MasR, is much higher in the heart, the kidney, the vasculature, and other tissues, reducing the plasma levels of ang-II and/or AT_1R activation by ACE inhibitors and selective antagonists, respectively, has been the focus to tackle RAS hyperactivity and treat various renal and cardiovascular diseases, including hypertension. Interestingly, however, RAS story seems to be more complex than ever before, particularly in light of new findings as to how the AT_2R and MasR may be joining forces together to oppose and counterbalance the deleterious effects mediated by the AT_1R. Purpose of this review is to highlight recent discoveries on AT_2R and MasR heterodimerization as a potential mechanism responsible for these receptors to amplify their cellular signal impacting RAS physiology related to cardiovascular function and BP regulation.

Role of the AT_2R and the MasR in Blood Pressure Regulation

AT_2R Activation and Signaling in Blood Pressure Control

AT_2R is an atypical G-protein (guanine nucleotide-binding protein)-coupled receptor (GPCR) with only 30% homology with AT_1R. Both the receptors are activated by ang-II with similar affinity [12]. Other studies have suggested ang-III as the preferred peptide agonist for AT_2R [13]. It is unusual that activation of AT_2R is linked to inhibitory ($G_{\alpha i/o}$) [14] as well as stimulatory ($G_{\alpha s}$) protein and even G-protein-independent pathways [15, 16]. It is the SH2 domain which predominately mediates AT_2R signaling via nitric oxide (NO)-cyclic guanosine monophosphate (cGMP) pathway [16], a pathway known to cause vasodilatation and natriuresis. Additionally, the AT_2R is linked to activation of tyrosine phosphatases. Recently, the AT_2R has been crystallized which provides a glimpse as to the chemical nature of the receptor [17]. One notable is the feature that AT_2R tends to stay in an active state without exposing with an agonist, a concept held based on early pharmacological and biochemical studies [18]. Specifically, mere increase in the AT_2R expression reduces the AT_1R function in terms of reducing inositol hydrolysis [10]. Consistently, the AT_2R is designated as an endogenous AT_1R antagonist. Moreover, unlike typical GPCRs, the AT_2R is resistant to agonist-induced desensitization, endocytosis, and degradation [19] due to fewer serine residues and the inability of the receptor to recruit β-arrestin [17]. The agonist

treatment triggers rapid transport of cytosolic AT_2R onto the plasma membrane which is likely to intensify AT_2R-mediated signaling and cellular response [20]. Collectively, these chemical and cellular regulatory features of the AT_2R make AT_2R an attractive potential target for agonist-based therapy. With the availability of a selective orally active AT_2R agonist compound 21 (C21), it became easier to study the AT_2R for its biological roles in health and disease.

The AT_2Rs are expressed in various organ systems including the heart, the vasculature particularly on endothelial cells, the brain, immune cells, and kidney tubular cells. Generally, the expression of AT_2R is upregulated under various pathological conditions both in humans and animal models. For example, human diabetic resistance arteries [21] and failing heart [22], as well as rodent diabetic [23] and obese [24, 25] kidney, express higher levels of the AT_2Rs. Information linking the AT_2R to natriuresis and BP control including inflammation and oxidative stress comes from knockout as well as pharmacological studies. Mice lacking the AT_2R exhibit modest higher BP [4] and higher sensitivity to ang-II elevating systolic BP [3, 4]. Also, these mice are more susceptible to deoxycorticosterone acetate (DOCA)-salt hypertension [26, 27]. Since the AT_2R-null mice express higher levels of the AT_1R [1], it is difficult to clearly ascribe the changes in natriuresis and BP directly due to lack of the AT_2R or increased the AT_1R expression/signaling. Although some studies show that the AT_2R-mediated natriuretic, vasodilatory, and depressor responses can be observed only in the presence of blockade of the AT_1R [28, 29] or ACE [30], other studies demonstrate that the AT_2R agonists such as peptide CGP42112a and nonpeptide C21 are able to produce natriuresis [24]. In obese Zucker rats, acute infusion of CGP42112a [24] or C21 [31] produced remarkable natriuresis. It is interesting to note that the AT_2R agonist does not affect natriuresis in control lean Zucker rats [24]. Similarly, other studies show enhanced natriuretic response in female spontaneously hypertensive rats (SHRs) (not in males) upon activation of AT_2R by the agonist C21 [32]. The greater response in obese Zucker rats (compared to lean) or females (compared to males) may be linked to the higher expression of the AT_2R in the kidney [23–25, 33]. Likewise, greater AT_2R function in females may be attributed to a positive feedback loop between the AT_2R expression and estrogen levels [34]. In the long term, chronic treatment with C21 of obese Zucker rats with high salt-induced hypertension [35•] or of Sprague-Dawley rats with ang-II-induced hypertension prevented increase in systolic BP to almost normal levels [36••]. In both the studies, C21 co-administration with high salt or ang-II produced greater natriuresis compared to high salt or ang-II alone [35•, 36••], preventing sodium and water retention and body fluid buildup thus preventing rise in BP. Central role of the AT_2R receptors in BP control also has been reported. Cerebroventricular infusion of the AT_2R agonist C21 lowers BP in normal rats [37••] and suppresses sympathetic outflow in failing heart model by improving baroreflex sensitivity [38]. Collectively, emerging studies provide strong evidences as to the role of AT_2R in fluid homeostasis and BP control/regulation. However, given that the AT_2R expression is very low compared with the AT_1R, it is puzzling as to how the AT_2R activation produces significant biological responses particularly under pathological conditions such as obesity, diabetes, salt-sensitive hypertension, inflammation, and oxidative stress. This review highlights, in the following section, that interaction with the AT_1R and MasR may be a plausible explanation to the AT_2R function.

MasR Activation and Signaling in Blood Pressure Control

The MasR is a proto-oncogene, which based on conserved structural motifs is classified as a GPCR and with ang-(1–7) as its natural agonist MasR belongs to the RAS. Multiple G-proteins such G_s, G_i, and G_{12} have been suggested to couple with the MasR [39], but vasodilatory NO and prostaglandins are the most reported signaling pathways linked to the MasR stimulation in the vasculature [40], heart [41, 42], and kidney [43]. Unlike the AT_2R, the MasR in response to its agonist ang-(1–7) stimulation internalizes through clathrin- or caveolin-1/dynamin-coated endocytic pit and slowly re-sensitizes through recycling protein Rab11. However, surprisingly, the internalized MasR does not follow classical lysosomal trafficking for degradation [44]. Another study shows that chronic treatment of normotensive Wistar-Kyoto (WKY) rats with ang-(1–7) does not affect the MasR expression in the kidney, but cardiac MasR expression is decreased [45], suggesting an organ/cell-type-specific regulation of the MasR. More studies are needed to understand the cellular regulation of MasR in response to chronic agonist exposure, particularly whether MasR degradation is tissue/cell specific. Similar to the AT_2R, the MasR also is considered a physiological antagonist of the AT_1R [11], i.e., the activation of the MasR produces natriuretic [46] and vasodilatory responses in various vascular beds [47–49], increases blood flow [50], and lowers BP in animal models of hypertension [8, 51, 52]. A recent article provides a thorough review on ang-(1–7), AVE0991, and CGEN-856S on vasodilation and antihypertensive activity of these MasR agonists [53]. Some of the early studies indicate that ang-(1–7) infusion produces natriuretic and diuretic responses and increases glomerular filtration rate [54–56]. Ang-(1–7) attenuates ang-II-stimulated Na^+-ATPase activity in isolated proximal tubules, suggesting a direct action on tubular sodium transport [57]. Recently, we have reported that ang-(1–7) produces natriuretic and diuretic responses which are blocked by the MasR antagonist A-779 [58••]. Other studies also confirmed natriuretic role of ang-(1–7) [59, 60], but there is some evidence suggesting antidiuretic actions of ang-(1–7) blocked by MasR antagonist D-ala^7-ang-(1–7) [61]. Similarly, some studies suggest that ang-(1–7) despite with a vasodilatory response does not lower BP [50, 62, 63] or increases mean arterial pressure [64–67], while other studies clearly demonstrate that acute as well as chronic infusion of agonists ang-(1–7) [68, 69], AVE0991 [70–72], or CGEN-856S [73] lowers BP in control SHRs and protected against BP elevation and end-organ damage provoked by L-N^G-nitroarginine methyl ester treatment in these animals [74].

Most of the initial discoveries assigned to the MasR function come from ang-(1–7) as an agonist, which through MasR knockout studies has been established as a MasR's natural agonist [75]. However, emerging evidences demonstrate that ang-(1–7) may not be binding to and eliciting its responses via MasR alone. For example, ang-(1–7)-elicited responses are attenuated by the AT_1R antagonist losartan [51, 59, 76] or the AT_2R antagonist PD123319 [76, 77, 78]. Several possibilities including heterodimerization of MasR and AT_2R with AT_1R (discussed in the next section) have been suggested in order to explain ang-(1–7) physiological functions. However, recent studies are of particular importance showing that ang-(1–7) also is a biased ligand for the AT_1R [79, 80••]. The ang-(1–7) binds to AT_1R and activates only β-arrestin pathway while blocking G_i/G_o-linked pathways and thereby produces cardioprotective effects mediated by AT_1R [79, 80••]. While there still exist questions related to the specificity of ang-(1–7) with MasR activation and the net responses,

more selective ligands for MasR, such as AVE0991 and CGEN-856S as agonists and A779 as antagonist, have been helpful to confirm and study the MasR-mediated physiological responses, as described above.

Dimerization and Functional Interdependence of the AT_2R and the MasR

As the AT_2R and MasR are components of the protective arm of the RAS, a communication in terms of functional interaction has been reported in many studies. For instance, the MasR agonist ang-(1–7) induced vasoprotective and atheroprotective effects in apolipoprotein E-deficient mice [77], amelioration of right ventricular modelling in diabetic rats [78•], and vasodepressor response in SHR and WKY rats [68] which are prevented by the AT_2R antagonist PD123319. Conversely, in monocrotaline-induced rat model of pulmonary hypertension, the AT_2R agonist C21 reduced pulmonary and ventricular fibrosis which is prevented by MasR antagonist A-779 [81]. Ang-(1–7) and C21 have been shown to improve neurological deficits in endothelin-1-induced stroke model which is cross-inhibited by their antagonists PD123319 and A-779 [82]. Such cross-inhibition indicates that these receptors may have post-receptor signaling cross talk or may exist in close proximity which allows them to interact at receptor level by heterodimerization. Recent two studies emerged at the same time showing that the AT_2R and the MasR make heterodimers that may be responsible for functional interdependence of these two receptors [58••, 83••]. Using gold standard co-immunoprecipitation and dual-immunolabelling experiments, we have reported an interaction of the AT_2R and the MasR in obese Zucker rat kidney and human kidney-2 (HK-2) cells [58••]. Moreover, incubation of renal cortical homogenate with cupric-phenanthroline (CuP), an oxidative cross-linker of proteins via free thiol (–SH) group of amino acids located within < 7 Å, followed by western immunoblotting of receptor complexes reveals that the AT_2R and the MasR bands on the blot shifted at higher molecular weight. This suggests a heterodimerization of AT_2R and MasR through free thiol groups of cysteine residues that may have located in very close proximity [58••]. This AT_2R-MasR interaction is sensitive to reducing agent, β-mercaptoethanol, suggesting that it is the disulfide formation that mediates the heterodimerization. Moreover, it seems that the AT_2R-MasR constitutively exists in the heterodimer form, as the western blotting in the absence of β-mercaptoethanol (not CuP either) reveals upward shift of some of the AT_2R and MasR; CuP converts 100% of the AT_2R-MasR into heterodimer or heteromers of the higher order. Earlier, through mutagenesis experiments, disulfide formation within the AT_2R has been shown to confer stability to AT_2R and introduction of free –SH groups leads to inactive population of the AT_2R [84], highlighting the importance of cysteine residues in the AT_2R activity. Another study utilized fluorescence resonance energy transfer (FRET) and cross-correlation spectroscopy in HEK-293 cells transfected with vectors encoding fluorophore-tagged AT_2R and MasR. The tagged receptors results in FRET with 10.8% efficiency, suggesting AT_2R and MasR are capable of forming heterodimers and dimerization disappears when AT_2R is mutated at Cys^{35} residue [83••]. Furthermore, Leonhardt et al. [83••] also show that the AT_2R and the MasR in astrocytes with CX3CR1 mRNA as the functional readout are interdependent. Specifically, blocking with either of the receptor antagonists (AT_2R, PD123319, and MasR, A-779) or knocking out of a single of these receptors made astrocytes unresponsive for both agonists. Consistent to these findings, our in vivo renal function experiment reveals that natriuretic and diuretic responses to the AT_2R

agonist C21 or MasR agonist ang-(1–7) were cross-inhibited by their selective antagonists [58••]. In light of reports that AT_2R [35•, 85••] and MasR [8, 86••] activations exhibit antihypertensive activity, it remains unknown whether blocking one receptor would impair the antihypertensive activity of the other receptor and to what extent.

Functionally important thiol group-containing amino acids are sensitive to oxidative stress and the CuP experiment provided cues that oxidative stress may play a critical role in dimerization of the AT_2R and the MasR. The treatment of HK-2 cells with glucose (25 mM), a known pro-oxidant molecule, followed by CuP reaction shows enhanced cross-linking of the AT_2R and the MasR (unpublished observations). Since obese Zucker rats exhibit modest hyperglycemia, consistent to the observation in HK-2 cells exposed to higher glucose concentration, there is an increase in the AT_2R-MasR dual-labelled puncta in the kidney of obese Zucker rats compared to lean Zucker rats (Fig. 1a, b). However, the increased dual labelling in obese rat kidney also could be due to the higher AT_2Rs expressed in obese rat kidneys [24]. In another study, we have reported that the AT_2R agonist chronic treatment of obese Zucker rats increases MasR expression [9], whether this would lead to more dimerization and enhanced the AT_2R function is not known. Overall, studies to determine whether a correlation exists between the levels of the AT_2R and MasR expression, heterodimer formation, and enhanced signaling and functional interdependence would have patho-physiological significance.

Kidney sections of obese rats and HK-2 cells also show the AT_2R-MasR puncta in the cytosol and on nuclear envelop, in addition to the plasma membrane. It appears that much of the AT_2R and the MasR are localized on organelles such as Golgi apparatus, mitochondria, and endoplasmic reticulum. The AT_2R [24] and MasR [87], both are heavily glycosylated and it is known that only completely glycosylated receptor is delivered to plasma membrane. It is likely that considerable amount of these receptors escape glycosylation and may remain in cytosol.

It has been reported that Golgi membrane-associated AT_2R-binding protein binds to specific motif within the cytoplasmic carboxy-terminal of AT_2R and enables its plasma membrane transport and anti-proliferative effects [88]. Carey's group also have reported that most of the renal AT_2Rs are located within the cytosolic compartments and translocate to the plasma membrane upon agonist exposure of the proximal tubules [20]. Whether it is the dimer that translocates to the plasma membrane in response to the AT_2R agonist and thus enhances the cellular signaling and response remains unknown.

Co-localization of the AT_2R-MasR is also observed in hypothalamic paraventricular (PVN) region of high-fat diet-fed male C57BL/6j mice (Fig. 2). The hypothalamus is an integrative center for numerous physiological events and hypothalamic RAS not only is involved in cardiovascular homeostasis and fluid balance [89, 90•] but also is implicated in neurohumoral [91], behavioral [92], cognitive [93], and metabolic aspects [94].

Interactions of the AT_2R and the MasR with the AT_1R

Since the AT_2R and the MasR have been designated as functional antagonist of the AT_1R, it is important to discuss their interaction, highlighting the functional complexity that seems to

exist. The AT_2R interacts with the AT_1R at both on the plasma membrane and intracellularly. Several reports show ligand-independent physical interaction between the AT_2R and the AT_1R and their functional cross talk [10, 18, 95, 96]. The AT_2R and the AT_1R exists as homodimers as well as heterodimers [18, 95]. It is not clear whether the AT_2R inhibits the AT_1R function via homodimerization and post-receptor signaling [96]. However, the role of heterodimerization of the AT_2R with the AT_1R is evidently shown to inhibit the AT_1R function, independent of AT_2R activation or signaling [10]. Upon upregulation of the AT_2R, it may heterodimerize with and inhibit the AT_1R. For instance, normotensive pregnancy is linked to higher AT_2R-AT_1R dimers as compared to pre-eclampsia wherein the AT_1R predominates [97]. Thus, it can be reasoned that the AT_2R-AT_1R heteromer is protective and the number of the AT_2R-AT_1R heteromer as compared to their respective monomer may regulate BP. However, ligand stimulation has been recently shown to enhance the AT_2R-AT_1R heteromerization resulting in change of conformation, co-internalization, and subsequent endosomal sorting of the AT_2R with AT_1R heteromer [98••] reducing the exposure of the AT_1R to its ligand ang-II. Besides, the AT_2R-AT_1R heteromer may reduce availability of extracellular ang-II levels by inducing ang-II internalization as a complex with the AT_2R-AT_1R heteromer [99]. And intracrine RAS may counteract and modulate deleterious effects of extracellular paracrine RAS [100]. Activation of the intracrine AT_1R is associated with increase in the expression of the AT_2R which may increase the AT_2R-AT_1R heteromers at the cell surface and initiate positive feedback loop. These findings provide new insights into an important regulatory aspect of the AT_2R in reducing the ang-II/AT_1R signaling at the cell surface. Conversely, prolonged exposure of the AT_1R to its ligands (ang-II, AT_1R auto-antibody) has been associated with reduced AT_1R internalization, sustained AT_1R signaling, and hypertension [101].

The MasR has been shown to constitutively heterodimerize with the AT_1R and to interfere with the ang-II/AT_1R signaling [11]. The constitutively active MasR through activation of $G_{q/11}$ may sort the AT_1R to the Golgi apparatus [102], plausibly reducing the ang-II-AT_1R signaling. Likewise, the MasR is also internalized upon stimulation; however, it is not known whether the MasR is co-internalized with the AT_1R [44]. Moreover, the pharmacologic activation of the MasR reduces the expression of the AT_1R in the kidney [103, 104]. The native MasR-AT_1R interactions [105] as well as opposing influence of the ang-(1–7)/MasR on the ang-II/AT_1R effects have been reported [76]. The vasodepressor effects of ang-(1–7) is unmasked in the presence of the AT_1R antagonist losartan, while ang-(1–7) increases perfusion pressure in the presence of the AT_2R antagonist PD123319 [76] and this increase is not sensitive to the MasR antagonist A779. This suggests a shift in ang-(1–7) functional behavior when the AT_2R has been blocked. Overall, it may be suggested that, in addition to monomers of the MasR, AT_1R, and AT_2R, a native existence of the oligomeric complex of MasR-AT_1R-AT_2R is a likely scenario.

Perspective

Dimerization of receptors can remarkably alter their ligand-binding properties, conformation, activation, and trafficking, which has (patho)physiological relevance [10]. In an essence, dimerization of RAS receptors still is an ill-defined, yet a central event in cross talk among these receptors and is increasingly being appreciated for the regulation of

receptor function. Based on above discussion, the existence of angiotensin receptors as subset in the forms of monomer, dimer, or higher-order oligomer (Fig. 3) is a likely phenomenon, which appears to be dependent on the cellular distribution, density of the individual receptors, and pathological versus normal conditions. Unfortunately, members of GPCR undergo dimerization in uncontrolled manner. Thus, screening for disease-specific receptor complexes and of receptor complex-specific dimeric ligands should be part of the future drug discovery targeting hypertension and associated cardiovascular morbidities.

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• Of importance

•• Of major importance

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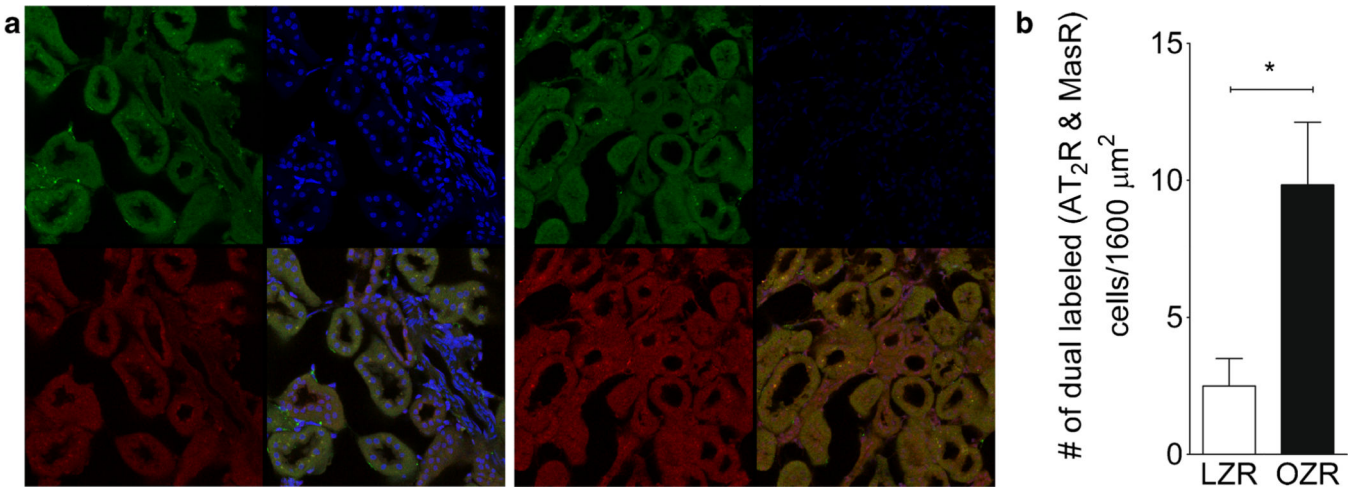

Fig. 1.

a The immunostaining of MasR (green, upper left) and AT_2R (red, lower left) with nuclear stain DAPI (blue, upper right) and merged image showing their colocalization (yellow puncta, lower right) in kidney of lean (left) and obese (right) Zucker rat and **b** the number of their dual-labelled puncta

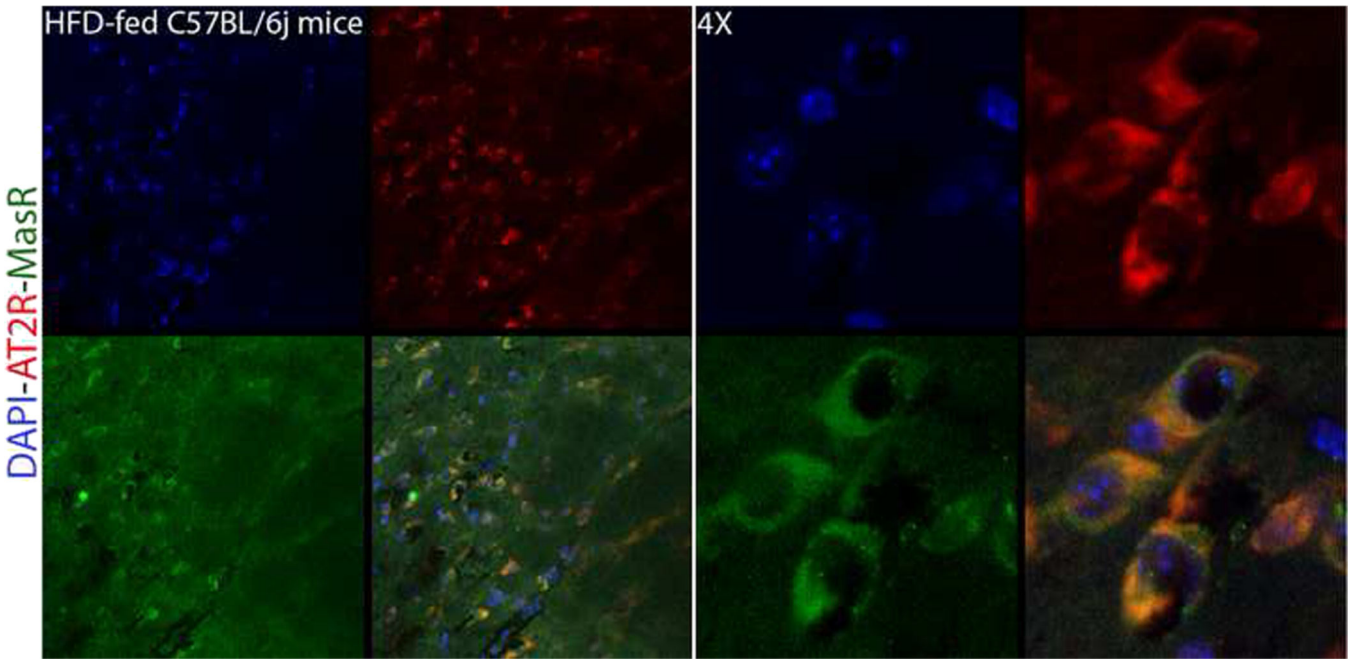

Fig. 2.

The co-localization of AT_2R and MasR in paraventricular region of hypothalamus of C57BL/6j mice fed high-fat diet for 8 weeks

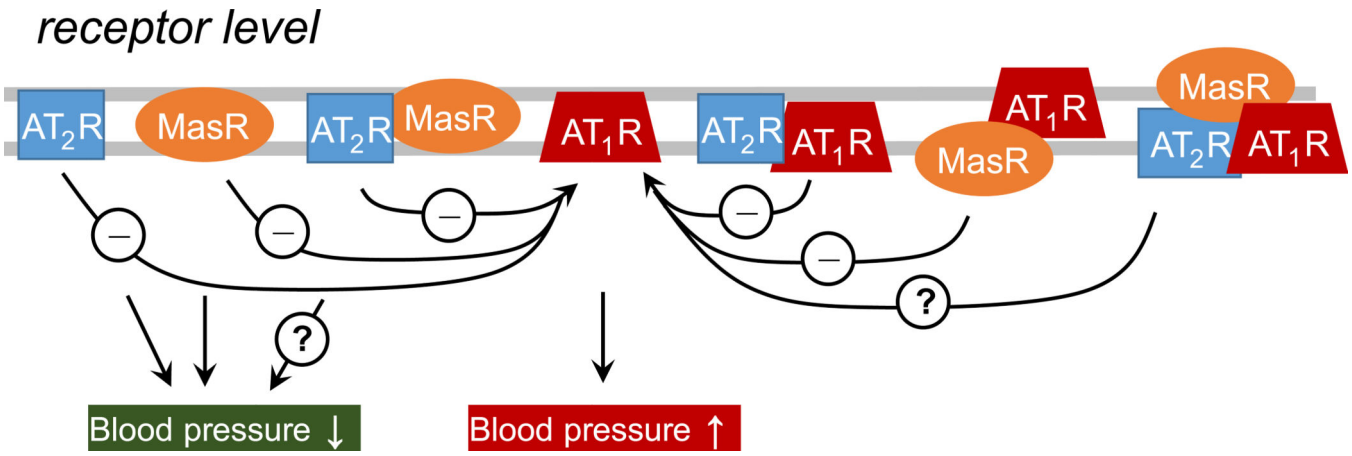

Fig. 3.

The proposed plausibilities among AT_2R, MasR, and AT_1R to interact in regulation of blood pressure