The Biological Roles of Angiotensin II in Target Tissues, G-Protein Mediated Signaling Mechanisms, Activation of the Jak / STAT Signaling Pathway and Stimulated ROS Generation and in Cancer and Autoimmunity Treatment and Prevention

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Abstract

Angiotensin II (Ang II) activates a wide spectrum of signaling responses via the AT1 receptor (AT1R) that mediate its physiological control of blood pressure, thirst, and sodium balance and its diverse pathological actions in cardiovascular, renal, and other cell types.

New signaling mechanisms, including G protein-dependent and -independent effects of AT1R activation, are emerging that begin to explain the unexpectedly broad clinical significance of the RAS.

Ang II-induced AT1R activation via Gq/11 stimulates phospholipases A2, C, and D, and activates inositol Tris-phosphate/Ca2 signaling, protein kinase C isoforms, and MAPKs, as well as several tyrosine kinases (Pyk2, Src, Tyk2, FAK), scaffold proteins (G protein-coupled receptor kinase-interacting protein 1, p130Cas, paxillin, vinculin), receptor tyrosine kinases, and the nuclear factor-kB pathway.

AT1R-mediated overproduction of reactive oxygen species has potent growth promoting, pro-inflammatory, and pro-fibrotic actions by exerting positive feedback effects that amplify its signaling in cardiovascular cells, leukocytes, and monocytes.

While targeted molecular therapies have considerably improved the management of primary breast tumors, these remain poorly effective for the treatment of distant metastases. The identification of molecular agents that may contribute to breast cancer cell dissemination and colonization is therefore essential for future development of new anti-metastatic therapeutic strategies.

In this article, I discuss Biological Roles of Local Ang II Formation in Target Tissues, Classical G-Protein Mediated Signaling Mechanisms, G Protein Independent Effects of Ang II Activation of MAPKs, Activation of the Jak /Signal Transducer and Activator of Transcription (STAT) Signaling Pathway, Modulation of AT1R Function Homo and Hetrodimerization and Ang II-Stimulated ROS Generation.

Key Word: Biological Roles of Ang II, the Renin-Angiotensin System, G-Protein, MAPKs, Jak Signaling, STAT Signaling Pathway, AT1R, and ROS

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1. Introduction

Angiotensin II, the octapeptide hormone that mediates most of the known biological actions of the RAS, was isolated in 1940 by Braun-Menendez *et al*. (1), and Page and Helmer (2). The action of renin on its substrate, angiotensinogen, generates the inactive decapeptide, angiotensin I, which is hydrolyzed by angiotensin- converting enzyme (ACE) to the potent pressor octapeptide, angiotensin II (Ang II). The RAS was initially regarded as an endocrine system in which circulating Ang II regulates blood pressure and electrolyte balance via its actions on vascular tone, aldosterone secretion, renal sodium handling, thirst, water intake, sympathetic activity, and vasopressin release. The cellular Ang II receptor was identified in 1974 as a high-affinity plasma membrane-binding site that is sensitive to guanyl nucleotides (3). Later, distinct

AT1 and AT2 receptor subtypes were identified by selective ligands (4), and were subsequently characterized as seven transmembrane receptors by molecular cloning (5),(6),(7),(8). The G protein-coupled AT1R mediates the known physiological and pathological actions on AngII and undergoes rapid desensitization and internalization after agonist stimulation. In contrast, the AT2 receptor does not exhibit the latter features and acts mainly through Gi and tyrosine phosphatases to exert predominantly inhibitory actions on cellular responses mediated by the AT1R and growth factor receptors (9). Recent studies using gene targeting to delete or enhance specific components of the RAS have confirmed its physiological roles and its novel functions in the development and maintenance of renal vascular and papillary structure (10),(11),(12). They have also highlighted the importance of paracrine and autocrine effects of local RAS in several tissues. Clinical trials using ACE inhibitors and AT1R blockers have shown that the actions of this system extend far beyond blood pressure control (13). It has become clear that the deleterious actions of local RAS on cardiovascular remodeling and renal function account for the beneficial effects of these agents in patients with hypertension, left ventricular hypertrophy, heart failure, and diabetic nephropathy (14). The RAS has also been proposed to exert intracrine effects due to the intracellular formation of Ang II (14),(15). However, kinetic studies suggest that intracellular Ang II is largely derived from receptor-mediated uptake of the extracellular peptide (16). Animal models and clinical data have also helped to establish that inhibition of Ang II action in nonclassical target sites, such as immune cells, explains some of the unanticipated therapeutic effects of ACE inhibitors and AT1R blockade (13). Parallel studies on the molecular mechanism of action of Ang II have revealed that its main target, the AT1R, is one of the most versatile members of the G protein-coupled receptor (GPCR) family. Ang II exerts several cytokine-like actions via the AT1R and can stimulate multiple signaling pathways, transactivate several growth factor receptors, and promote the formation of reactive oxygen species (ROS) and other proinflammatory responses. Furthermore, recent evidence indicates that the AT1R may serve as a "stretch sensor" because mechanical stress can activate the AT1R without the involvement of Ang II (17),(17). Many of these effects are highly cell specific, and numerous studies are currently under way to identify the roles of these signaling pathways in the physiological and pathophysiological actions of Ang II (9),(18),(19),(20).

2. The Renin-Angiotensin System

The classical RAS is an enzymatic cascade initiated by the cleavage of circulating angiotensinogen by renin, an aspartyl protease, to form the decapeptide angiotensinI. Angiotensin I is further cleaved by ACE, a dipeptidyl carboxypeptidase, to produce the (21), circulating Ang II octapeptide that is the main effector hormone of the RAS. The ectoenzyme, ACE, is expressed at the surface of endothelial cells, where it catalyzes the conversion of Ang I to Ang II. ACE also cleaves and inactivates bradykinin and thus contributes to the therapeutic effects (and side-effects) of ACE inhibitors. The ACE gene has an insertion/deletion polymorphism in which a 287-bp deletion in intron 16 (ACE-D) leads to increased tissue and circulating levels of the mutant enzyme. This deletion has been reported to be a risk factor for certain cardiovascular and renal diseases and the progression of sarcoidosis (22),(23). The first evidence for the peripheral formation of Ang II came from studies on angiotensin metabolism in the sheep (24). The octapeptide hormone was subsequently found to be produced in numerous tissues, including the adrenal, brain, heart, kidney, vasculature, adipose tissue, gonads, pancreas, prostate, eye, and placenta (12),(25),(26),(27). Although not all components of the classical RAS are synthesized locally in some tissues, alternative enzymatic pathways or the

presence of the renin receptor, which binds to and activates circulating renin and prorenin, may also permit local Ang II formation. Ang II can be further metabolized by aminopeptidase A to form Ang III [Ang(2–8)] and then by aminopeptidase N to Ang IV [Ang(3–8)]. Ang II and III exert their effects via the AT1R and AT2R. Ang IV is also a biologically active peptide with low affinity for AT1R and AT2R and acts in the brain on insulin-regulated aminopeptidase. This protein is a transmembrane metalloprotease that cotranslocates in vesicles with glucose transporter 4 to the cell surface in response to Insulin (28). Ang IV and the endogenous ligand, LVVhemorphin 7, which are potent inhibitors of insulinregulated aminopeptidase, enhance memory and learning and reverse memory deficits in amnesic animals (29). A recently identified second form of ACE (ACE-2) and other endopeptidases can cleave Ang I to produce Ang(1–7), which has vasodilator and cardioprotective effects (20). Ang(1–7) lacks the C-terminal phenylalanine8 residue of Ang II that is critical for binding and activation of AT1Rs, and has been proposed to act via another GPCR, putatively the mas oncogene (30).

3. Biological Roles of Local Ang II Formation in Target Tissues

The beneficial effects of ACE inhibitors and AT1R blockers on the morbidity and mortality of patients with cardiovascular diseases are at least partially independent of the blood pressure-lowering effects of these drugs (13),(31),(32). Furthermore, such effects are also observed in patients with low renin hypertension (33), and the pharmacokinetic properties of ACE inhibitors suggest that their principal actions occur at tissue sites (34). Discrepancies between the circulating renin and Ang II levels, and the results of clinical studies with drugs that inhibit the RAS, have indicated the importance of local Ang II formation in various tissues (12), Moreover, the functions of the tissue and systemic RAS activities show significant differences. The circulating Ang II concentration is relatively low (10–50 pM) and, even in pathological situations, rarely reaches the level required for 50% saturation of the AT1R (25). However, classical Ang II target tissues, such as adrenal glomerulosa cells and vascular smooth muscle cells (VSMCs), contain abundant AT1Rs and exhibit adequate intracellular signaling to activate biological responses at the relatively low levels of receptor occupancy that pertain at circulating hormone levels. Furthermore, in many tissues the locally formed Ang II levels are much higher than the plasma concentration (16), and can elicit significant biological responses in cells with relatively low levels of AT1R expression. Thus, the importance of the nonclassical Ang II target tissues is particularly relevant when the local generation of the peptide is sufficiently high.

4. Classical G-Protein Mediated Signaling Mechanisms

The AT1R is a typical heptahelical GPCR and, on agonist binding, elicits multiple cellular responses, predominantly via coupling to Gq/11, and also to G12/13 proteins, and Gi/o in rodents (4). Gq/11-mediated inositol phosphate/Ca2_ signaling is the primary transduction mechanism initiated by Ang II in its major physiological target tissues, including adrenal, neuronal, cardiac, renal, and smooth muscle cells (4). The pathophysiological importance of this pathway for Ang II-induced hypertension and cardiac hypertrophy was demonstrated in mice expressing a C-terminal Gq peptide that inhibits receptor/Gq/11 interaction (35). The AT1R can also couple to pertussis toxin-sensitive Gi/o proteins that inhibit adenylyl cyclase in some target tissues (*e.g.* rat adrenal glomerulosa, liver, kidney, and pituitary cells) (4). In adrenocortical cells, Gi/o- mediated actions of Ang II also contribute to its regulatory actions on voltage-sensitive T- and L-type Ca2_ channels (25),(36),(37),(38). Another important mechanism

is activation of the G11/12 family of G proteins, which contribute to the physiological actions of Ang II by mediating its activation of phospholipase C (PLC) and phospholipase D, L-type Ca2_ channels, and Rho kinase (39),(40),(41),(42). Ang II-induced stimulation of cAMP responses has also been reported, but this results from activation of Ca2-sensitive adenylyl cyclases or calcineurin, a Ca2-dependent phosphatase, rather than receptor coupling to Gs (43). In addition to its rapid, G protein-mediated actions via second messengers and their pathways, Ang II activates secondary responses that are typically associated with stimulation of growth factor receptors, and regulate cell growth, proliferation, cell migration, apoptosis, and gene expression. These effects are often the consequence of Ang II-induced activation of cytoplasmic tyrosine kinases such as Pyk2, c-Src, Tyk2, FAK, Janus kinase 2 (Jak2), and transactivation of membrane-associated growth factor receptor tyrosine kinases (44),(45). Ang II-induced cytoplasmic Ca2_ signaling and protein kinase C (PKC) activation regulate the activities of Src, FAK, and Pyk2 (45). Activation of heterotrimeric G proteins by the AT1R also releases their - subunits, which can cause further activation of tyrosine kinases (46),(47). At least one tyrosine kinase (Jak2) has been shown to interact directly with a tyrosine-containing motif in the cytoplasmic tail of the AT1R (48). Tyrosine phosphorylation of receptors and scaffold proteins, such as growth factor receptors, integrins, GPCR kinase-interacting protein 1 (GIT1) and p130Cas, paxillin, tensin, vinculin, Grb2 and other adapters, is an important factor in the organization of signaling pathways (45),(49). For example, PLC_ is constitutively associated with GIT1, a GPCR kinaseinteracting protein, and Ang II-induced, Src-mediated tyrosine phosphorylation of GIT1 leads to its activation (50). Although Ang II-induced stimulation of inositol triphosphate signaling is primarily caused by G protein- mediated activation of PLC_ isoenzymes, tyrosine kinase-regulated activation of PLC_ also makes a significant contribution to this process in certain cell types (*e.g.* VSMCs) (4). In addition, GIT1 can serve as a scaffold protein to facilitate Ang II- and epidermal growth factor (EGF)-induced activation of MEK (MAPK kinase 1) and ERK (51), A recent study suggests that GIT1 is a scaffold for ERK1/2 activation in focal adhesions and regulates cell migration in VSMCs and other cells (52). Another recently identified, Src-regulated scaffold protein is p130Cas, which associates with focal adhesions and regulates the migration of smooth muscle cells via activation of c-Jun NH2-terminal kinase (JNK) (49). Ang II is a potent stimulus of smooth muscle growth and migration and extracellular matrix formation in the vascular system (53). It stimulates the proliferation of human cerebral artery VSMCs through a basic fibroblast growth factor-dependent pathway, but causes hypertrophy without proliferation in VSMCs from peripheral arteries (54). Ang II also stimulates the proliferation of cultured human mesangial cells through activation of MAPKs, specifically JNK (55), and of IEC-18 intestinal epithelial cells via transactivation of the EGF receptor (EGFR) and the phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin/p70S6K1 signaling pathway (56). In adrenal glomerulosa cells, for which Ang II acts as a trophic factor *in vivo* as well as a stimulus to aldosterone secretion, variable effects on growth and proliferation have been observed *in vitro*, in(51) some cases with stimulation via calcineurin and MAPK (57). However, a recent report indicates that Ang II stimulates protein synthesis but inhibits proliferation in cultured rat adrenal glomerulosa cells (58). In the testis, Ang II stimulates contraction and growth of the peritubular myoid cells, apparently via MAPK and tyrosine phosphorylation (59). In the skin, activation of AT1R signaling promotes wound healing by stimulating the growth of keratinocytes and myofibroblasts (60). In many tissues, stimulation by Ang II leads to MAPK activation by transactivating growth factor receptors, including EGF, platelet-derived growth factor, and IGF receptors, and Axl (61),(62),(63), but in some cells [*e.g.* human embryonic kidney

(HEK) 293 cells], PKC activation can directly activate the pathway leading to ERK activation (64). Transactivation of the EGFR and other growth factor receptors is an important component of AT1R-mediated signaling via MAPK pathways in cardiac and smooth muscle cells. This transactivation pathway is mediated by Ca2_, PKC, and Src-dependent transactivation of transmembrane matrix metalloproteases, which cause release of heparin- binding EGF from its precursor in the cell membrane and activation of the EGFR (65),(66),(67). In hepatic C9 cells Ang II-induced activation of endogenous AT1Rs mediates ERK1/2 activation by a similar mechanism (64),(68),(69). In C9 cells, agonist activation of endogenous AT1R and EGFR induced the caveolindependent formation of a multireceptor complex containing both the AT1R and the transactivated EGFR. Conversely, activation of EGFR caused phosphorylation, internalization, and desensitization of AT1Rs, via a PLC_, PI3K-, and PKC-mediated mechanism (70). Many of the effects of Ang II-induced signal transduction pathways on gene expression are mediated by the activation and nuclear translocation of MAPKs. The detailed mechanisms of Ang II-induced, AT1Rmediated activation of ERK1/2, JNK, p38 MAPK, and ERK5 in specific tissues are described in earlier reviews (4),(25),(45),(71). More recent developments, including the roles of G protein-independent mechanisms and ROS in these processes, are discussed below.

5. G Protein Independent Effects of Ang II Activation of MAPKs

The agonist-activated AT1R can also stimulate G protein- independent signal transduction mechanisms by directly associating with signaling molecules, such as -arrestins and tyrosine kinases. Arrestins were initially defined as molecules that bind to agonist activated and phosphorylated GPCRs and cause homologous desensitization of these receptors by uncoupling them from their cognate G proteins. Like many other activated GPCRs, the AT1R binds -arrestins, which have clathrin and activator protein (AP) 2 adapter-binding domains that target them to endocytosis via clathrin-coated vesicles. Recent studies also demonstrated that -arrestins can also serve as proteins that organize signaling complexes for the activation of MAPKs (72),(73),(74),(75),(76). As in other GPCRs, activation of the AT1R leads to its phosphorylation by specific receptor kinases and binding of -arrestin, which are uncouples it from G proteins and also serves as an adaptor for clathrin-mediated internalization. The AT1R belongs to the class B group of GPCRs, which bind -arrestins tightly due to the pres-ence of a defined serine-threonine-leucine motif (77),(78), in their cytoplasmic tail (79),(80). This tight binding causes -arrestin to remain associated with the receptor during its internalization into endosomes (76). Arrestins also serve as scaffolds to organize the formation of signaling complexes (73), that mediate AT1Rmediated activation of ERK and JNK3 MAPKs (76). Studies on a mutant AT1R that cannot couple to G proteins have shown that _-arrestin2-mediated ERK activation can occur in the absence of G protein activation (81). Although the biological role of this process has not been defined, it could promote the phosphorylation of cytosolic targets of ERK, which when activated by this mechanism cannot enter the nucleus due to their association with the receptor, and are mostly located on the surface of endosomes (73). Studies on PAR2 receptors have shown that -arrestin-mediated MAPK activation does not stimulate DNA synthesis or cell division but can promote reorganization of the actin cytoskeleton and chemotaxis and may contribute to migration of cancer cells(82),(83),(84). A recent report suggests that Ang II can also induce chemotaxis by a G protein-independent, -arrestin-mediated mechanism (85). Additional studies are needed to elucidate the exact biological function(s) of these mechanisms after AT1R activation. Mutations in the highly conserved Asp125Arg126Tyr127 sequence of the AT1R result in a mutant receptor that is fully uncoupled

from G proteins, but can still activate Src tyrosine kinases in Chinese hamster ovary-K1 cells (86). The G protein-independent activation of Src by this mutant receptor can cause Ras-dependent ERK 1/2 activation, which however fails to phosphorylate nuclear targets. Recently, Gq protein independent, AT1R-mediated EGFR transactivation was also reported to occur in human coronary artery smooth muscle cells (87). The role of -arrestins in these pathways needs clarification, because, as noted above, under similar conditions AT1R can mediate –arrestin mediated ERK activation in HEK-293 cells (81). Furthermore,-arrestin-dependent complex formation between Src tyrosine kinase and the _2-adrenergic receptor, but not the AT1R, has been reported (88). Studies with another mutant AT1R, with deletions of Ala221 and Leu222, suggest that Ang II-induced, Cdc42-mediated activation of JNK can occur in a mutant receptor that is unable to activate Gq-mediated signaling (89),(90). The latter mutant was also able to stimulate Rap1-dependent ERK1/2 activation, but the physiological relevance of this pathway needs further studies because it was not activated by the wild-type receptor.

6. Activation of the Jak/Signal Transducer and Activator of Transcription (STAT) Signaling Pathway

In cardiac fibroblasts, aortic smooth muscle cells, and the kidney, Ang II-induced AT1R activation also stimulates the Jak/STAT pathway, a characteristic signal transduction mechanism of cytokine receptors (5). This pathway has been linked to several of the pathophysiological effects of Ang II, including cardiac hypertrophy, dysfunction and heart failure (91), vascular inflammation and smooth muscle cell growth (92), and renal tubulointerstitial fibrosis (93). Jak/STAT activation also provides a positive feedback pathway by up-regulating angiotensinogen formation, leading to increased production of Ang II (94). The ability of cyclical stretch to induce matrix metalloproteinase expression in cardiac myocytes is also mediated by the Ang II-Jak/STAT pathway (95). However, Ang II exerts both direct and indirect actions on the Jak/STAT pathway because AT1R activation also causes the release of IL-6 and other cytokines that stimulate this pathway. Although Ang II clearly serves as a proinflammatory mediator by acting on VSMCs and endothelial cells, and also by directly activating macrophages and other immune cells, the exact role of Ang II-induced activation of the Jak/STAT pathway in immune cells has not yet been defined. Janus kinases (Jaks) are cytoplasmic tyrosine kinases initially identified as essential components of interferon receptor signaling (96). The Jak family includes three ubiquitously expressed members (Jak1, Jak2, and Tyk2) and Jak3, which is expressed primarily in hematopoetic cells. During cytokine stimulation, activation of Jak kinases leads to tyrosine phosphorylation and activation of STAT proteins, which dimerize and translocate to the nucleus to regulate the expression of target genes. The Jak/STAT signal transduction pathway can also be activated by GPCRs, and Ang II-mediated activation of STAT1–3, STAT5, and STAT6 has been reported (91),(97),(98). The exact roles of the individual STAT proteins are currently under investigation, and it appears that activation of STAT1 and STAT3 in cardiac cells exerts opposing biological actions by inducing 1) apoptosis, proinflammatory, and antiproliferative mechanisms; and 2) cardioprotective, mostly antiinflammatory and Ang II-Stimulated AT1Rs Also Activate G Protein- Independent Signaling Pathways growth-stimulatory responses, respectively (91). Interestingly, most of these GPCRs, including receptors for Ang II, thrombin, chemokines, and platelet activating factor, are found on the surface of blood cells, where the Jak/STAT pathway has a major role in cytokine signal transduction. In VSMCs, the AT1R activates Jak2 via its association with a YIPP motif in the C terminal tail of the receptor (48). The same motif is required for PLC-_ activation (99), and

possibly for EGFR transactivation (100), and optimal binding of the AT1R (101). Studies in VSMCs have suggested that Ang II-induced tyrosine phosphorylation of Jak2 is G protein mediated, because it is dependent on increased cytoplasmic Ca2_ concentration and activation of PKC (102). However, other studies with mutant receptors indicate that the AT1R can also activate the Jak/STAT pathway via G protein-independent mechanisms. Substitutions of five carboxyl-terminal tyrosine residues (Tyr292, Tyr302, Tyr312, Tyr319, and Tyr339) with phenylalanine, and substitution of Asp74 with glutamic acid, led to mutant AT1Rs that were unable to activate Gq, but could induce tyrosine phosphorylation and activation of Jak2 and its effector, STAT1 (103). Such activation of the Jak/STAT pathway could stimulate cell growth in the absence of Gq- mediated signaling (103). These data suggest that the AT1R can regulate intranuclear targets via the Jak/ STAT pathway in the absence of G protein activation.

7. AT1R-Associated Proteins

The C-terminal cytoplasmic domain of GPCRs binds to a variety of intracellular proteins involved in receptor signaling, desensitization, and endocytosis. These include proteins such as GRKs and _-arrestins, and receptor-specific proteins that bind to individual receptors or classes thereof. Those associated with the AT1R include PLC and Jak proteins (48),(99). and novel proteins found by two-hybrid studies, including AT1R-associated protein (ATRAP) (104), Ang II receptor- associated protein 1 (ARAP-1)(105), EP24.15 (EC 3.4.24.15, thimet oligopeptidase) (106), and GLP, a GDP/GTP exchange factor-like protein(107). (108). PLC and Jak2 kinase [the latter possibly via Src homology phosphatase 2 (SHP-2) as an adaptor] have been found to associate with the YIPP sequence in the proximal region of the cytoplasmic domain(48),(99). ATRAP is an 18-kDa protein with three N-terminal transmembrane domains and interacts specifically with the C-terminal region of the AT1R (104), Endogenous and transfected ATRAP is located in the plasma membrane and within intracellular compartments including endoplasmic reticulum, Golgi, and endocytic vesicles (108). Furthermore, it is associated with the AT1R at both sites and appears to influence receptor endocytosis and function in diverse cell types. In cardiomyocytes, overexpression of ATRAP reduces cell surface AT1Rs, as well as Ang II-induced signaling and protein synthesis (109). ARAP-1 is a less well-characterized 57-kDa protein that may have a role in AT1R recycling to the plasma membrane (105). An interesting and recently identified AT1R-associated protein is the novel 531-residue protein termed GLP, which stimulates hypertrophic responses when expressed in VSMCs and immortalized renal proximal tubule cells (107). The AT1R also associates with caveolin in smooth muscle cells, and this has been proposed to coordinate Ang II-induced signaling (110). Other studies have suggested that a caveolin-bindinglike motif in the receptor's cytoplasmic tail has an important role in the recycling of internalized AT1Rs to the plasma membrane (111). Recently, caveolin was found to be essential for Ang II-stimulated targeting of the AT1R into lipid rafts and activation of Rac1, and subsequent ROS-mediated hypertrophic responses in VSMCs (112).

8. Modulation of AT1R Function Homo and Hetro-dimerization

Recent studies using fluorescence or bioluminescence resonance energy transfer have demonstrated that many GPCRs exist in the cell as dimers or oligomers. Although the stoichiometry of this process (*i.e.* dimers or oligomers) is difficult to assess with these biophysical techniques, a recent study on the organization of rhodopsin using atomic force microscopy suggest that rhodopsin-related, family A GPCRs, such as the AT1R, exist in dimers that may be further arranged into oligomeric arrays (113). Homo- and heterodimerization of several GPCRs have been reported (114). The biological function of this process is most evident for family C GPCRs, where heterodimerization is required for proper targeting of newly synthesized receptors to the cell surface. Agonist-independent homodimerization of transiently expressed AT1Rs has been detected by bioluminescence resonance energy transfer studies in transfected COS-7 cells, in which dimerization of the receptor occurs during its early biosynthesis before cell surface expression (115),(116). This is consistent with a role of this process in targeting receptors to the cell surface, as reported for other GPCRs. Studies on the coexpression of mutant AT1Rs suggest that this process may have biological significance for the function of cell surface receptors. Also, the coexpression of two mutant AT1Rs with impaired Ang II binding affinity rescues the ligand binding activity of the native receptor (117). The coexpression of binding (K199A) and G protein coupling-impaired mutant (D125E/ R126E/Y127A/M134A) receptors with the wild-type AT1 receptor has a dominant-negative inhibitory action on its inositol phosphate response, without affecting its agonist-induced _-arrestin and ERK activation (116). These data are compatible with the hypothesis that AT1Rs, like rhodopsin, can exist and function as dimers. Recently, a new mechanism and biological function for AT1R dimerization has been reported in monocytes, where cross-linking of AT1R dimers by factor XIIIA transglutaminase appears to promote enhanced signaling and desensitization *in vitro* and *in vivo*(118). Elevated levels of cross-linked AT1Rs were observed in hypertensive patients, and a role of this process in the onset of atherosclerosis was proposed (118). Additional studies are needed to confirm the general relevance of these interesting findings. The AT1R also forms heterodimers with the AT2R, and with the B2 bradykinin and -adrenergic receptors (119),(120),(121). Studies on the AT1R and other GPCRs indicate that, although heterodimerization can occur between several pairs of GPCRs, it is a specific process that involves well-defined partners (114). Heterodimerization of the AT1 and B2 receptors results in increased activation of Gq and Gi proteins and changes the endocytic pathway of the receptor (119). Although the biological function of this process is not yet known, hetero-dimerization of these receptors was reported to occur *in vivo* in platelets and omental vessels and has been proposed to be related to the increased AT1R responsiveness in preeclampsia (122). Association of the AT1R with the AT2R inhibits its signaling activity and function (120), which may contribute to the antagonistic biological effects of the AT2R on the AT1R (see above). However, it is not yet known whether the AT2R has similar inhibitory actions on other GPCRs. This process merits further investigation, because it may explain the previously observed ligand-independent actions of the AT2R on apoptosis (123). However, it is important to emphasize that not all of the anti-AT1R effects of the AT2R are attributable to the heterodimerization of these receptors. The AT2R can activate phosphotyrosine and serine/threonine phosphatases that inhibit MAPK activation, including MAPK phosphatase 1, protein phosphatase 2A, and SH2 domain-containing phosphatase- 1 (9). It also stimulates the formation of bradykinin, NO, and cGMP, which cause vasodilation and exert antiproliferative actions in vessels and attenuate AT1R-mediated cardiac fibrosis and anti-natriuretic effects in the kidney (18). Recent studies on the physical association of AT1Rs and adrenergic receptors have clearly demonstrated the potential pharmacological importance of GPCR hetero-

dimerization, because interactions between the two receptors lead to the formation of molecular complexes in which blockade of the AT1R can inhibit signaling via _-adrenergic receptors, and *vice versa* (121). Elucidation of the functional role and biological significance of the hetero-dimerization of AT1 with other GPCRs will require further investigation.

9. Ang II-Stimulated ROS Generation

The production of ROS and activation of redox-sensitive signaling pathways mediate many of the actions of Ang II. Under physiological conditions, ROS-dependent redox-signaling processes contribute significantly to the normal cellular responses to Ang II and other humoral mediators. However, excessive accumulation of ROS causes oxidative stress leading to cellular damage or cell death. This is an important factor in the pathophysiological proinflammatory, profibrotic, and mitogenic actions of Ang II and leads to endothelial dysfunction, angiogenesis, vascular and cardiac remodeling during the development of hypertension, atherosclerosis, cardiac and smooth muscle hypertrophy, vasculitis, and diabetes (124). ROS are produced as intermediates during redox processes that lead to the formation of water from oxygen. The many enzymes that catalyze such reactions include reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, nitric oxide synthases, xanthine oxidases, cytochrome P450s, mitochondrial respiratory chain enzymes, cyclooxygenases, lipoxygenases, and myeloperoxidases. NADPH oxidases are the major source of ROS in leukocytes, but some of the latter enzymes are also important in endothelial cells. The univalent reduction of molecular oxygen by a free electron leads to the formation of highly reactive but unstable superoxide, which only crosses membranes via anion channels. Some of superoxide's effects result from its rapid reaction with vasodilator and cytoprotective nitric oxide to form highly reactive, toxic peroxinitrate. Superoxide is also converted by dismutases to hydrogen peroxide, which is membrane permeant and has a longer half-life than superoxide. This facilitates the spread of the oxidative signal or stress to other intracellular compartments. Hydrogen peroxide is scavenged by catalase and gluthatione peroxidase but can also be reduced to generate highly active hydroxyl radicals in the presence of metal-containing molecules (*e.g.* Fe2)(125). ROS exert their effects by oxidative modification of target molecules, *e.g.* oxidation of cysteine residues. This is exemplified by the inactivation of protein tyrosine phosphatases by oxidation of conserved cysteines in their active sites, which catalyze the hydrolysis of protein phosphotyrosine residues by forming a cysteinyl-phosphate intermediate (126). This shifts the balance between tyrosine kinases and phosphatases toward phosphorylation, potentiating the effects of Ang II and other humoral mediators that activate tyrosine kinases. ROS also targets numerous other signaling molecules, including ion channels, MAPKs (JNK, p38 MAPK, ERK5), matrix metalloproteinases, and transcription factors [nuclear factor-kB (NFkB), AP1] (125). In VSMCs, Ang II-induced activation of JNK, p38 MAPK, and ERK5 is ROS-sensitive, whereas ERK1/2 activation is not (124). By these mechanisms, ROS promotes the activation of numerous signaling pathways, some of which further stimulate ROS production and exert positive actions on pathways that amplify signaling. Under physiological conditions, these pathways are controlled by antioxidant mecha-nisms, but in pathological states such excessive ROS accumulation causes progressive cell damage. Ang II induces ROS generation in many target tissues, and this appears to result largely from activation of NADPH oxidases. This occurs in VSMCs (127), endothelial cells (128),(129), cardiac cells (130), phagocytic cells (131), and fibroblasts (132). NADPH oxidase was first characterized in neutrophils, where it consists of two membrane-associated (gp91phox and p22phox) and four cytosolic (p40phox, p47phox, p67phox and Rac)

subunits (125). In phagocytic cells, phosphorylation of p47phox initiates the assembly of the enzyme complex and causes the respiratory burst by binding to p22phox. Homologs of gp91phox (Nox2) are also present in nonphagocytic cells. Nonphagocytic NADPH oxidases include NOX1, NOX3–5, and two related proteins, DUOX1 and DUOX2, which have NADPH oxidase and peroxidase activity. NOX1 is regulated by NOX organizer 1 and NOX activator 1, which are homologous to p47phoxand p67phox, suggesting common principles in the assembly of phagocytic and nonphagocytic NADPH oxidases (133). Endothelial cells express NOX1, 2, 4, and 5, whereas VSMCs express NOX1, 4, and 5, and possibly NOX2 (124). During the rapid and biphasic stimulation of ROS production by Ang II in rat aortic VSMCs, an initial PKC-dependent response during the first minute of agonist stimulation is followed by a slower second increase caused by transactivation of the EGFR (134). In accordance with the role of EGFR transactivation during the sustained phase of Ang II-induced ROS generation in VSMCs, inhibition of EGFR transactivation (by AG1478 or Src kinase inhibitors) or of EGFR-mediated ROS production (by inhibitors of Rac or PI 3-kinase) diminishes the sustained increase of ROS generation. Thus, AT1R-mediated activation of Rac can mediate ROS formation, in addition to its previously reported role in JNK activation (135),(136). Studies by Touyz *et al*. (137),(138), have also implicated Src kinase and phospholipase D in Ang II-induced ROS generation in VSMCs. However, it is uncertain whether these effects are mediated directly by the AT1R or result from transactivation of the EGFR or other growth factor receptors. The NADPH oxidase that mediates Ang II-induced ROS formation in VSMCs has yet to be identified. Ang II appears to induce the phosphorylation and membrane association of p47phox, leading to activation of the phagocytic NADPH oxidase, NOX2 (138). This is consistent with findings that Ang II does not induce activation of superoxide production in murine VSMCs and endothelial cells from p47phox-deficient mice (139),(140). However, Ang II-dependent atherosclerosis in the descending aorta of apolipoprotein E-deficient mice is diminished in offspring from these animals crossed with p47phox mice, but not with NOX2 mice (141),(142). These data indicate that the pathophysiological effects of p47phox can be mediated by nonphagocytic NADPH oxidases. Furthermore, NOX2 can produce rapid burst-like ROS release in the extracellular (phagosomal) space, whereas NOX1 and NOX4 generate low-level, predominantly intracellular ROS both constitutively and in response to agonists such as Ang II(124). In VSMC, NOX1 and NOX4 are localized in caveolae and focal adhesions, respectively, which determine the differential roles of these enzymes because NOX1 mainly participates in cell growth, whereas NOX4 induces apoptosis and senescence (143). Ang II also stimulates the formation of ROS in human neutrophils, leading to activation of MAPKs that may contribute to the pathophysiological effects of the RAS during inflammation (131). Such Ang II-induced ROS formation is mediated by activation of the AT1R via a PI 3-kinase-dependent pathway, which is also facilitated by ERK1/2 and p38 MAPKs. Ang II also increases cytosolic Ca2_ levels and stimulates calcineurin and NFkB activities. Although it is clear that Ang II can regulate the function of neutrophils, further studies are required to elucidate the role(s) of AT1Rmediated second messenger generation in ROS formation and to identify the mechanisms caused by autocrine/paracrine effects of IL-6 and other cytokines released by Ang II.

10. Conclusion

The scope and biological significance of angiotensin's numerous actions, and the roles of critical signaling mechanisms that are susceptible to dysfunction leading to progressive inflammatory and degenerative conditions that underlie major disease entities. The application of therapeutic approaches based on this recently acquired knowledge is facilitating the amelioration or abolition of many of the deleterious effects of inappropriately activated Ang II/AT1R signaling pathways, which are now known to contribute to several disease states. It is generally accepted that blockade of the local generation of Ang II is a key factor in the clinical benefits of ACE inhibition and AT1R antagonists. Ang II can activate cytokine signaling pathways in cardiovascular cells and is emerging as a typical cytokine that can regulate immune cells and inflammatory processes. However, the exact signal transduction mechanism of Ang II in leukocytes and macrophages, and the biological roles of AT1R dimerization and G protein-independent signaling pathways, need additional investigation and further elucidation.

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