Angiotensin II cell signaling: physiological and pathological effects in the cardiovascular system

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Mehta PK, Griendling KK. Angiotensin II cell signaling: physiological and pathological effects in the cardiovascular system. Am J Physiol Cell Physiol 292: C82–C97, 2007. First published July 26, 2006; doi:10.1152/ajpcell.00287.2006.—The renin-angiotensin system is a central component of the physiological and pathological responses of cardiovascular system. Its primary effector hormone, angiotensin II (ANG II), not only mediates immediate physiological effects of vasoconstriction and blood pressure regulation, but is also implicated in inflammation, endothelial dysfunction, atherosclerosis, hypertension, and congestive heart failure. The myriad effects of ANG II depend on time (acute vs. chronic) and on the cells/tissues upon which it acts. In addition to inducing G protein- and non-G protein-related signaling pathways, ANG II, via AT1 receptors, carries out its functions via MAP kinases (ERK 1/2, JNK, p38MAPK), receptor tyrosine kinases [PDGF, EGFR, insulin receptor], and nonreceptor tyrosine kinases [Src, JAK/STAT, focal adhesion kinase (FAK)]. AT1R-mediated NAD(P)H oxidase activation leads to generation of reactive oxygen species, widely implicated in vascular inflammation and fibrosis. ANG II also promotes the association of scaffolding proteins, such as paxillin, talin, and p130Cas, leading to focal adhesion and extracellular matrix formation. These signaling cascades lead to contraction, smooth muscle cell growth, hypertrophy, and cell migration, events that contribute to normal vascular function, and to disease progression. This review focuses on the structure and function of AT1 receptors and the major signaling mechanisms by which angiotensin influences cardiovascular physiology and pathology.

vascular smooth muscle; NAD(P)H oxidase; tyrosine and nontyrosine receptor kinases; endothelial dysfunction; vascular disease

THE RENIN-ANGIOTENSIN SYSTEM (RAS) plays a vital role in regulating the physiological processes of the cardiovascular system. Not only does it function as an endocrine system, but it also serves local paracrine and autocrine functions in tissues and organs. The primary effector molecule of this system, angiotensin II (ANG II), has emerged as a critical hormone that affects the function of virtually all organs, including heart, kidney, vasculature, and brain, and it has both beneficial and pathological effects. Acute stimulation with ANG II regulates salt/water homeostasis and vasoconstriction, modulating blood pressure, while chronic stimulation promotes hyperplasia and hypertrophy of vascular smooth muscle cells (VSMCs) (53, 216). In addition, long-term exposure to ANG II also plays a vital role in cardiac hypertrophy and remodeling, in-stent restenosis, reduced fibrinolysis, and renal fibrosis.

Given its diverse range of functions and its potency in affecting cardiovascular physiology, it becomes imperative to understand the characteristics of ANG II receptors and to investigate mechanisms of ANG II-induced signal transduction. Studying the varied roles of ANG II is also of tremendous importance given the beneficial effects of angiotensin converting enzyme inhibitors (ACE-I) and angiotensin receptor blockers (ARBs) in reducing morbidity and mortality in diabetes, hypertension, atherosclerosis, heart failure, and stroke (69, 77, 168, 220). This review focuses on the structural and functional characteristics of ANG II receptors, the major ANG II-induced cell signaling cascades, and their role in cardiovascular physiology and pathology.

THE RENIN-ANGIOTENSIN SYSTEM

The mechanisms controlling the formation and degradation of ANG II are important in determining its final physiological effect. An octapeptide, ANG II is formed from enzymatic cleavage of angiotensinogen to angiotensin I (ANG I) by the aspartyl protease renin, with subsequent conversion of ANG I to ANG II by angiotensin converting enzyme (ACE). A recently identified carboxypeptidase, ACE2, cleaves one amino acid from either ANG I or ANG II (29), decreasing ANG II levels and increasing the metabolite Ang 1–7, which has vasodilator properties. Thus the balance between ACE and ACE2 is an important factor controlling ANG II levels (32). Even though ACE is the primary enzyme leading to ANG II generation, in the heart the majority of ANG I is converted by chymase (213). Nguyen and colleagues (130) have recently shown that activation of the renin receptor also increases the conversion of angiotensinogen to ANG I, with resultant activation of mitogen-activated protein kinases (MAPKs); interestingly, a high level of renin receptor mRNA is present in the heart and renin receptors have been detected in the subendothelium of coronary and renal arteries. The tissue-specific
effect of increased ANG II levels and enhanced RAS activity depends on the cellular expression and activation of AT1Rs, critical receptors in cardiovascular and renal pathophysiology.

AT1 RECEPTORS

Structural Characteristics

Most of the known physiological effects of ANG II are mediated by angiotensin type 1 receptors (AT1Rs), which are widely distributed in all organs, including liver, adrenals, brain, lung, kidney, heart, and vasculature. Composed of 359 amino acids, the AT1R (40 kDa) belongs to the seven-membrane superfamily of G protein-coupled receptors. The human AT1R gene has been mapped to chromosome 3. In rats, two isoforms that share 95% amino acid sequence identity have been identified: the AT1aR on chromosome 17 and the AT1bR on chromosome 2 (61). Functionally and pharmacologically, the two receptor subtypes are indistinguishable (52); however, in vivo experiments show that the AT1aR isoform may be more important than AT1bR in regulation of blood pressure (25). The extracellular domain of the receptor is characterized by three glycosylation sites, and mutation of these sites has no effect on agonist binding. G protein interactions occur on the transmembrane domain at the NH2 terminus and the first and the third extracellular loops (23). Along with several residues located on the extracellular region of the receptor, four cysteine residues of AT1R form disulfide bridges and are essential for ANG II binding (143). Similar to other receptors (muscarnic and adrenergic), the AT1 receptor’s cytoplasmic tail contains many serine/threonine residues, which are phosphorylated by G protein receptor kinases or GRKs (discussed later). Modifications within these functional sites may be responsible for the altered receptor function in cardiovascular disease.

Polymorphisms

Genetic variations in the RAS cascade have been associated with cardiovascular disease. Evidence suggests that genetics play an important role in interindividual differences in response to ANG II. Recent advances in gene mapping have identified single nucleotide polymorphisms (SNPs) of the AT1R gene that have been linked to an increased development of cardiovascular risk factors. The A1166C polymorphism of the AT1R gene has been implicated in hypertension (21), increased aortic stiffness (14), and myocardial infarction (16). One study in hypertensive patients on a high-salt diet found an association between A1166C polymorphisms and increased ANG II sensitivity (179). In isolated human arteries, A1166C LDL (132) Angiotensin II (65) Erythropoietin (12) Vitamin A (185)
Progesterone (135) Estrogen (135)
Insulin (184) Interferon-γ (78)
HMG CoA reductase inhibitors (75)
Platelet-derived growth factor (133)
HMG, 3-hydroxy-3-methyl-glutaryl-

Table 1. Regulation of AT1Rs in the cardiovascular system

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Agonists That Upregulate</th>
<th>Agonists That Downregulate</th>
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<tbody>
<tr>
<td>VSMCs</td>
<td>LDL (132)</td>
<td>Angiotensin II (65)</td>
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<tr>
<td></td>
<td>Insulin (184)</td>
<td>Interferon-γ (78)</td>
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<td>Progesterone (135)</td>
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<td>Erythropoietin (12)</td>
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<td>HMG CoA reductase inhibitors (75)</td>
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<td></td>
<td></td>
<td>Epidermal growth factor (66, 199)</td>
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<td></td>
<td>Platelet-derived growth factor (133)</td>
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<td></td>
<td></td>
<td>Thyroid hormone (49)</td>
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<td></td>
<td></td>
<td>Nitric oxide (76)</td>
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<td></td>
<td></td>
<td>Forskolin (62)</td>
</tr>
</tbody>
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References are given in parentheses. VSMCs, vascular smooth muscle cells; HMG, 3-hydroxy-3-methyl-glutaryl.
Desensitization and Internalization

Similar to many agonist-receptor systems, the effect of ANG II on its target tissues appears to be transient; that is, after stimulation with this hormone, tissue is desensitized to further agonism. Experiments performed over a decade ago showed that AT1Rs are endocytosed within 10 min of its activation (60). Approximately 25% of the internalized receptors are recycled back to plasma membrane, and the remainder are degraded in lysosomes (60, 65). Numerous proteins appear to play a role in the highly coordinated process of endocytic recycling, including β-arrrestins, G protein-mediated phospholipase D2, and the Rab family of GTPases (37).

One of the mechanisms believed to play a part in receptor desensitization involves receptor phosphorylation. On the cytoplasmic surface of the AT1R, several serine/threonine phosphorylation sites serve as a substrate for GRKs, which mediate receptor desensitization by uncoupling the receptor from its activated G protein. In HEK-293 cells, GRK-2, GRK-3, and GRK-5 have been shown to phosphorylate the rat AT1R, preventing activation of protein kinase C (PKC) (145). Furthermore, in rat VSMCs, ANG II has also been shown to phosphorylate AT1Rs (88). Once the COOH terminus of AT1R is phosphorylated by GRK 2/3, the receptor is internalized into specialized, clathrin-coated pits (50, 105). This process is mediated by β-arrrestins, a group of multifunctional proteins that not only initiate receptor internalization, but also serve as scaffolds to link downstream signaling molecules to G protein-coupled receptors (92, 105). Interestingly, at physiologic concentrations of ANG II, internalization of AT1Rs into clathrin-coated pits is β-arrrestin dependent, but when AT1Rs are saturated with ANG II, their internalization is β-arrrestin independent (178). Once endocytosed, the receptor induces specific cell signaling pathways. For example, when AT1Rs are phosphorylated by GRK 5/6, β-arrrestin-mediated ERK signaling is activated, independent of G protein signaling (92). Defects in the desensitization process have been implicated in vascular disease; indeed, ANG II-induced hypertensive rats appear to overexpress GRK-5, altering ANG II responsiveness (82).

More recently, there is strong evidence that AT1R internalization also occurs via noncoated pits. After agonist binding, the AT1R moves to noncoated specialized microdomains called caveolae, associated with caveolin (83). Signaling molecules such as EGFR and Src are co-localized with caveolin-1 (Cav-1) found in these specialized microdomains (58, 224). In VSMCs, ANG II promotes the association of AT1R with Cav-1, and enables trafficking into caveolin-rich domains. The association of Cav-1 with AT1Rs appears vital in Rac 1 activation (224). Recently, it has also been demonstrated that when stimulated by ANG II, c-Src immediately activates c-Abl (205); activation of c-Abl allows for translocation of Rac 1 to lipid rafts, which in turn promotes NAD(P)H-dependent ANG II signaling, causing VSMC hypertrophy (224).

Heterologous regulation of AT1R and its endocytic recycling is only possible if the cellular trafficking and recycling systems tightly regulate and coordinate the transport of AT1Rs to the cell membrane. The Rab family of proteins are Ras-related GTPases that regulate intercellular vesicular transport. Specifically, Rab 1 has been associated with transport of AT1Rs from endoplasmic reticulum to Golgi to cell surface (214). Recent studies report that Rab 5 contributes to the trafficking and fusion of clathrin-coated vesicles with early endosomes (178). In COS-7 cells, the interaction of Rab5a with the COOH terminus of AT1R promotes its transport to enlarged endosomes (170). Compartmentalization of AT1Rs into these microdomains may be necessary for efficient signaling, considering the spatial relationships of different proteins.

AT2 RECEPTORS

Even though most of the vasoactive effects of ANG II occur via AT1Rs, AT2Rs have been shown to exert anti-proliferative and pro-apoptotic changes in VSMCs, mainly by antagonizing AT1Rs (61). Similar to the AT1R, the AT2R (MW 41 kDa) is a seven transmembrane domain receptor, but is only 34% identical to AT1R (127). Consisting of 363 amino acids, AT2R is highly expressed in fetal tissue, including fetal aorta, gastrointestinal mesenchyme, connective tissue, skeletal system, brain, and adrenal medulla. AT2R expression declines after birth, suggesting that it may play an important role in fetal development (175), and can be induced later in adult life under pathological conditions. Autopsy results of nonfailing human hearts show that the heart has approximately 50% AT2Rs; in chronic heart failure, AT1Rs are downregulated compared with AT2Rs (197). AT2Rs are also expressed at low levels in kidney, lung, and liver, but their exact role in carrying out the functions of ANG II remains undetermined. Studies have shown that AT2R antagonizes AT1R by inhibiting its signaling pathways via activation of tyrosine or serine/threonine phosphatases (13, 128). However, D’Amore and colleagues (31) recently found that AT2Rs cause hypertrophy in cardiomyocytes, independent of ANG II, and not block AT1R-mediated hypertrophy. This hypertrophic response is mediated by direct binding of the transcription factor PLZF (promyelocytic leukemia zinc finger protein) to the tail of the AT2R, leading to nuclear translocation and enhanced transcription of the p85 subunit of phosphatidylinositol 3-kinase (PI3K) (101, 173). In contrast, consistent with its antagonistic effects on AT1R, in a mouse model of inflammation-dependent vascular disease, deletion of AT2Rs enhanced neointimal formation and inflammation (23). Furthermore, dimerization of the two receptor types also causes an interruption in AT1R signaling (1). The exact role and the extent to which AT2Rs play a role in pathology (or are a consequence of pathology) is unclear as various studies have produced conflicting results.

ANG II SIGNALING PATHWAYS

Once ANG II binds to the AT1R, it activates a series of signaling cascades, which in turn regulate the various physiological effects of ANG II. Traditionally, the pathways induced by ANG II have been divided into two classifications: G protein- and non-G protein-related signaling; however, these distinctions are becoming blurred as more data emerge. One well established mechanism by which ANG II signaling occurs involves the classic G protein-mediated pathways. In addition to activating the G protein-dependent pathways, ANG II also cross-talks with several tyrosine kinases via AT1Rs, including receptor tyrosine kinases [EGFR, PDGF, insulin receptor and...
nonreceptor tyrosine kinases [c-Src family kinases, Ca\(^{2+}\) -dependent proline-rich tyrosine kinase 2 (Pyk2), focal adhesion kinase (FAK) and Janus kinases (JAK)]. In addition, many of ANG II’s pathologic effects in the vasculature occur via activation of NAD(P)H oxidases and generation of reactive oxygen species (ROS) (63). AT\(_{1}\)R also activates serine/threonine kinases such as PKC and MAPKs [including ERK1/2, p38MAPK, and c-Jun NH\(_2\)-terminal kinase (JNK)] that are implicated in cell growth and hypertrophy. The induction of the above mentioned pathways is tightly regulated; in patients with overstimulated RAS or enhanced responsiveness to ANG II, these pathways may initiate and propagate pathological events promoting vascular disease (73, 183).

The temporal and spatial patterns of signaling pathway activation are the most likely determinants of a particular functional response. Multiple studies show that the activation of different pathways by ANG II is time dependent. For example, activation of the G protein-dependent pathway and generation of IP\(_3\) occurs in seconds, while MAP kinase and JAK/STAT activation occurs in minutes to hours after initial activation of AT\(_{1}\)R (118, 169). Furthermore, differences in receptor/ligand affinity, alteration in trafficking patterns, AT\(_{1}\)R structural modifications, and the local tissue environment all appear to play a role in the ultimate effects of ANG II signaling.

**G Protein-Coupled Pathways**

One of the major acute functions of ANG II is vasoconstriction, which is mediated by “classical” G protein-dependent signaling pathways (see Fig. 1). Evidence shows that when activated by an agonist, AT\(_{1}\)Rs couple to G\(_{oq/11}, \) G\(_{12/13}, \) and G\(_{\beta\gamma} \) complexes (202), which activate downstream effectors including phospholipase C (PLC), phospholipase A\(_2\) (PLA\(_2\)), and phospholipase D (PLD) (200). Activation of PLC produces inositol-1,4,5-triphosphate (IP\(_3\)) and diacylglycerol (DAG) within seconds. IP\(_3\) binds to its receptor on sarcoplasmic reticulum, opening a channel that allows calcium efflux into the cytoplasm. Ca\(^{2+}\) binds to calmodulin and activates myosin light chain kinase (MLCK), which phosphorylates the myosin light chain and enhances the interaction between actin and myosin, causing smooth muscle cell contraction (217). To counter-regulate MLCK, cells have myosin light chain phosphatase (MLCP), which is inhibited by Rho kinase, leading to sustained contraction (85, 177). Evidence shows that inhibition of Rho kinase blocks Ca\(^{2+}\)-induced sensitization in smooth muscle cells (198). Furthermore, ANG II has also been shown to increase phosphorylation of CPI-17 (a MLCP inhibitor) via PKC (171). In addition, DAG activates PKC, which not only serves to increase the pH during cell contraction by phosphorylating the Na\(^+\)/H\(^+\) pump (206), but also participates as an effector in the Ras/Raf/MEK/ERK pathway. These downstream molecules contribute to the vasoconstrictive properties of AT\(_{1}\)R activation and lead to ANG II’s growth promoting effects. ANG II-induced G protein signaling may also explain the relationship between hyperglycemia and vascular dysfunction (55, 180). In VSMCs cultured from hyperglycemic rats, PKC inhibition attenuates ANG II-mediated growth and migration (219), both of which contribute to vascular lesion formation.

Agonist-AT\(_{1}\)R interaction also leads to PLD activation, resulting in hydrolysis of phosphatidylcholine (PC) to choline and phosphatic acid (PA). PA is rapidly converted to DAG, leading to sustained PKC activation, and sustained muscle contraction. The PLC/PLD pathways causing muscle contraction are augmented in hypertensive rats compared with controls, suggesting that alterations in the G protein-activated second messengers may play a role in the pathogenesis of hypertension (9, 194).

ANG II has been shown to phosphorylate and activate PLA\(_2\), which leads to production of arachidonic acid (AA) and its metabolites. The derivatives of AA function in maintaining vascular tone and in VSMC NAD(P)H oxidation (63). The cyclooxygenase-derived prostaglandins, such as PGI\(_2\) and PGE\(_2\) are vasodilatory, and are counteracted by PGH\(_2\) and NO, nitric oxide.

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**Fig. 1.** The role of ANG II in vascular smooth muscle cell contraction. Via G protein signaling, AT\(_{1}\)Rs are able to mobilize Ca\(^{2+}\) from the sarcoplasmic reticulum, and promote the interaction of actin and myosin filaments to allow for contraction and migration of cells. ANG II-induced arachidonic acid metabolism via phospholipase A\(_2\) also maintains a balance between vasoconstriction and vasodilation in various vascular beds. One of the major pathways activated by G protein interaction with AT\(_{1}\)R leads to the activation of PKC and the ERK pathway, implicated in sustenance of contraction as well as cellular growth. PC, phosphatidylinositol bisphosphate; PLA\(_2\), phospholipase A\(_2\); PA, phosphatic acid; PIP\(_2\), phosphatidylinositol bisphosphate; PLC, phospholipase C; PKC, protein kinase C; DAG, diacylglycerol; IP\(_3\), inositol trisphosphate; NO, nitric oxide; COX, cyclooxygenase; LT, leukotrienes; LO, lipoxygenase; TX\(_{A2}\), thromboxane A\(_2\); PG, prostaglandins; EET, epoxyeicosatrienoic acid; HETE, hydroxyeicosatetraenoic acid; LO, lipoxygenase; TX\(_{A2}\), thromboxane A\(_2\); NO, nitric oxide.
thromboxane A₂, which promote vasoconstriction. Via lipoxygenase, ANG II also mediates the formation of leukotrienes, implicated in vasoconstriction, hypertension, and inflammatory diseases. Arachidonic acid metabolites hydroxyeicosatetraenoic acids (HETEs) are pro-hypertensive, and lead to ANG II-mediated smooth muscle vasoconstriction by facilitating Ca²⁺ entry into the cell (164). These are counter-regulated by cytochrome P450-mediated epoxycosatrienioic acid (EETs) and dihydroxyeicosatetraenoic acids (DiHETEs), which are anti-hypertensive. EET- and DiHETE-mediated vascular relaxation appears to occur via inhibition of calcium-activated potassium channels (24).

Besides VSMC contraction, G protein-mediated pathways also activate various downstream proteins that further enhance growth and migration related signaling. The duration and intensity of signaling by the G protein subunits of AT₁R is mediated by members of a class of regulators of G protein signaling (RGS); in particular, RGS2 is a key player in inhibiting the Goα subunit and its subsequent actions. Grant and colleagues (57) showed that RGS2 mRNA is significantly upregulated within 24 hours of ANG II stimulation and this increase is partially PKC dependent. In RGS2-deficient mice, prolonged vasoconstriction in response to ANG II has been demonstrated. In addition, candesartan, an AT₁R antagonist, decreases blood pressure in these mice (70). Recently, it has been shown that the cardiovascular system differentially expresses RGS isoforms. Aorta contains RGS1–5, vena cava expresses RGS5, atria contain a high level of RGS1 and RGS2, while the left ventricle contains the highest level of RGS4 (3, 28). Of interest, RGS1–4 all attenuate ANG II/AT₁R signaling (28), and studying their role in vascular pathology warrants further research since they may be potential targets for therapeutic intervention.

NAD(P)H and ROS Signaling

Oxidative stress has been implicated in regulation of tyrosine kinases and phosphatases, expression of inflammatory genes, endothelial function, VSMC growth, and extracellular matrix formation (63, 153, 191, 219, 221). ANG II is a potent mediator of oxidative stress and oxidant signaling (186, 191, 201, 217). ANG II activates membrane NAD(P)H oxidases in VSMCs to produce ROS such as superoxide and hydrogen peroxide (H₂O₂), which are involved in the pleiotropic effects of ANG II (63, 153, 204, 221). The mechanism by which ANG II activates NAD(P)H oxidases remains under intense investigation. In aortic smooth muscle cells, the NAD(P)H oxidase subunits Nox1 and Nox4 are mainly responsible for ROS generation (103). ANG II-mediated activation of NAD(P)H oxidases involves the upstream mediators Src/EGFR/PI3K/Rac-1 (discussed below) and PLD/PKC/p47phox phosphorylation (174, 195).

Previously considered to be only toxic byproducts of metabolism, ROS are now known to be potent intercellular and intracellular second messengers that mediate signaling in pathways causing hypertension and vessel inflammation (63, 141). ROS such as H₂O₂ can reversibly modify cysteine residues and regulate activity of tyrosine phosphatases and peroxiredoxins (163). Superoxide can also modify heme groups and iron-sulfur centers on proteins, interfering with their function (8, 71). Many signaling molecules are now known to be ROS sensitive, and many ANG II-mediated effects are dependent on ROS. For instance, ANG II-induced activation of p38MAPK depends on H₂O₂; in VSMCs that overexpress catalase (an enzyme that catabolizes H₂O₂), p38MAPK activation is inhibited. Similarly, activation of Akt/PKB, Src, EGFR, and many others is also ROS sensitive. Transcription factors, such as NF-κB, AP-1, and Nrf2, which are implicated in the pathogenesis of atherosclerosis, are also activated by ROS (26, 147, 172, 215). One of the most well-established consequences of superoxide generated by ANG II is inactivation of nitric oxide (NO) in endothelial cells and VSMCs (64, 156). It has been shown that ROS also cause vessel inflammation by inducing release of cytokines and leukocyte adhesion molecules that increase recruitment of monocytes to the area of endothelial damage (121). Thus, interactions between ANG II signaling and ROS lead to changes in structural and functional characteristics of the vasculature and are critical in vascular pathology.

Mitogen-Activated Protein Kinases

Cellular protein synthesis and metabolism, transport, volume regulation, gene expression, and growth all depend on MAPks. ANG II has been shown to activate signaling cascades that activate MAPks, including extracellular signal-regulated kinase (ERK1/2), INK, and p38MAPK, which are implicated in VSMC differentiation, proliferation, migration, and fibrosis (see Fig. 2) (182, 188).

The ERK pathway is the best characterized of the MAPK pathways. Binding of ANG II to AT₁Rs activates ERK1/2 within 5 minutes, and in VSMCs ERK 1/2 activity is blocked by inhibition of PLC, suggesting its dependency on calcium (42). Src and calcium-dependent kinase Pyk2 phosphorylate EGFR on tyrosine, leading to formation of the Scf/Grb2 complex and ERK activation. This scaffold permits activation of Raf, which in turn phosphorylates the MAPK/ERK kinase (MEK). Raf associates with the small G protein Ras, leading to MEK activation, and subsequent phosphorylation of ERK 1/2 on threonine/tyrosine residues (182); recently, PKC-ζ has also been shown to associate with Ras and activate ERK 1/2 (108, 109). The phosphatase MAP kinase phosphatase-1 (MKP-1) serves as a negative feedback control, inactivating ERK 1/2 (20). Interestingly, stimulation of AT₂Rs activates phosphatases that also block ERK-mediated activity (30, 72).

Recent data implicates ERK (p42/44 kinase) in ANG II-mediated VSMC contraction. Touyz et al. (192) showed that in VSMCs from human peripheral arteries, tyrosine kinases and the ERK signaling cascade play a role in Ca²⁺ and pH pathways, which ultimately cause cell contraction; specifically, MEK/ERK may increase Ca²⁺ availability within cells. Of importance, PD98059 (an inhibitor of MEK) and Tyrphostin A-23 (tyrosine kinase inhibitor) attenuate contraction caused by ANG II (192). ERK has also been implicated in anti-apoptotic and pro-mitogenic effects, and activation of ERK1/2 and Akt/PKB has been shown to inhibit apoptosis (4, 111, 137). Furthermore, ERK 1/2 has been implicated in ANG II-induced cellular growth and protein synthesis via regulation of PHAS-1 (inhibitor of eukaryotic initiation factor 4E). Recently, it has been shown that Pyk2 is an upstream player in ANG II-mediated regulation of PHAS-1 via ERK 1/2 (155).

ANG II-mediated MAPK activation is followed by an increase in c-fos (activated by ERK) and c-jun (activated by...
JNK) gene expression, as well as increased AP-1 activity. AP-1 is a transcription factor complex (formed from dimerization of c-Jun and c-Fos) and ultimately influences cell differentiation, migration, and adhesion by binding to gene promoter sequences (191). ANG II-induced enhanced activation of vascular MAP kinases such as ERK1/2 has also been implicated in hypertension and in micro- and macrovascular target-organ damage (80, 81).

In addition to activating ERK1/2, ANG II also stimulates MAP kinases that are associated with environmental stress, such as apoptosis signal regulating kinase 1 (ASK1), which subsequently induces JNK and p38MAPK related signaling (74, 190). JNK (phosphorylated by MEK 4/7) and p38MAPK (phosphorylated by MEK 3/6) are amongst the family of stress-induced kinases that influence cell survival, apoptosis, and differentiation. JNK and p38MAPK have also become known as important mediators of ANG II-induced vascular inflammation (47, 98). Recently, it has been shown that ASK 1 is needed for ROS-induced JNK and p38MAPK activation (190), and inhibition of ASK 1 by thioredoxin leads to inhibition of apoptosis (114). Izumiya et al. (84) also discovered that in vivo, ASK 1 is vital in ANG II-induced cardiomyocyte hypertrophy and remodeling. In hypertensive rats, JNK pathways in vascular and renal tissues have also been implicated in vascular remodeling (94).

It has been discovered that unlike ERK and p38MAPK activation, ANG II-induced JNK activation is independent of EGFR transactivation (40). As shown by Nishida and colleagues (136), ANG II-stimulated activation of JNK and p38MAPK depends on Gα12/13-mediated activation of Rho/Rho kinase, with resultant activation of the small G protein Rac and ROS production. Activation of the JNK pathway by ANG II can also occur via Gq-mediated activation of PKC-δ, and subsequent stimulation of Pyk-2 and PDZ-RhoGEF-mediated Rho activation (142). Pathways downstream of Rac have been implicated in migration (171). In fact, the Rac effector p21-activated kinase (α-PAK), which is rapidly activated upon ANG II stimulation, has been identified as being upstream of JNK in VSMCs (169). ANG II-mediated activation of p38MAPK occurs via NAD(P)H oxidase-derived ROS (196); p38MAPK leads to stimulation of MAPKAPK-2, which serves to phosphorylate heat shock protein HSP-27, a stress-induced protein needed to chaperone and stabilize intracellular proteins (181, 188). In addition, p38MAPK also plays a role in ANG II-induced activation of Akt, a kinase with multiple downstream effects, including glucose metabolism and protein synthesis. These varied effects of ANG II-mediated MAPKs may provide more links between oxidant stress, hypertension, hyperlipidemia, and diabetes in the development of inflammation and atherosclerosis.

Nonreceptor Tyrosine Kinases

Src pathway. Even though AT1R lacks intrinsic kinase activity, nonreceptor tyrosine kinases associate with AT1R and initiate signaling events that lead to phosphorylation and activation of several intracellular proteins. In recent years, Src has surfaced as a key player in ANG II-mediated cellular effects (Figs. 2 and 3). c-Src is a tyrosine kinase that has been shown to be activated by Gαq, in a ROS-dependent manner, and it is involved in activation of a variety of downstream pathways, including Ras, FAK, JAK/STAT, and PLC-γ (leading to sustained calcium release). Src kinase and its substrates such as FAK and Pyk2 (also known as cell adhesion kinase-β) associate with paxillin, talin, and p130Cas to form a complex involved in JNK-mediated activation of AP-1 (see Fig. 3). Pyk2 activation by ANG II is dependent on Ca2+ and PKC in VSMCs (161), and further activates c-Src and 3-phosphoinositide-dependent kinase (PKD)-1, leading to cell growth and assembly of focal adhesion complexes (189). Interestingly, Ishida et al. (81) found that in VSMCs stimulated by ANG II, c-Src is involved in focal adhesion complex formation and actin bundling; furthermore, VSMCs lacking c-Src had less tyrosine phosphorylation of p130Cas, paxillin, and tensin. Important in focal adhesion signaling through the extracellular matrix, FAK and Pyk2 are rapidly phosphorylated by ANG II,
Fig. 3. ANG II promotes cell adhesion and extracellular matrix formation. A cell’s ability to survive with various environmental stressors is also regulated by kinases such as JNK, which has been found to be downstream of α-PAK and Rac, second messengers activated by AT₁R signaling. ANG II-induced association of FAK, Pyk2, paxillin, talin, and p130Cas forms a complex that promotes cellular adhesion and extracellular matrix synthesis via JNK activation. Evidence shows that calcium plays a vital role in mediating these ANG II-induced processes. JNK activation by ANG II can also occur via Gq and G₁₁₂, with RhoA/RhoA kinase activation. RGS, regulators of G protein signaling; ROS, reactive oxygen species; JNK, c-Jun NH₂-terminal kinase; FAK, focal adhesion kinase; GEF, guanine nucleotide exchange factor; PDK1, 3-phosphoinositide-dependent kinase 1; PKC, protein kinase C; PLD, phospholipase D; α-PAK, p21 activated kinase; AP-1, activator protein-1; ROCK, RhoA kinase.

further illuminating the critical cytokine-like properties of ANG II in mediating cellular growth (17). JAK/STAT pathway. The activated AT₁R also induces the JAK/STAT mitogenic pathway. Upon activation by JAK, STAT proteins dimerize via sulfhydryl-phosphotyrosine interactions, and translocate to the nucleus, where they mediate gene transcription of early growth response genes, such as c-fos and c-myc (17, 81, 117). It has been discovered that ANG II stimulates the association of JAK2 with AT₁R via tyrosine phosphatase SHP-2; the COOH terminus of AT₁R serves as a docking site for JAK2, and stimulates JAK2 phosphorylation at Tyr1007/1008 (48, 119). Frank and colleagues (48) showed that in VSMCs, PLC and its downstream second messengers, IP₃/Tyr1007/1008, plays a vital role in mediating these ANG II-induced processes. ANG II-activated JAK/STAT signaling pathways. ANG II-induced JAK2 activation. Furthermore, this activation is dependent on PKC-δ and Pyk2. ANG II-activated JAK/STAT signaling is blocked by SHP-1, a phosphatase that causes JAK2 dephosphorylation (120). Via JAK/STAT signaling, ANG II exhibits its multifaceted role in mediating VSMC growth, migration, and remodeling.

FAK and Pyk2 pathway. In response to various pathologic stimuli, cells reorganize their cytoskeletal structure to promote survival, migration, adhesion, and apoptosis. ANG II signaling through the cytoskeleton integrates many growth and redox signaling pathways. ANG II regulates formation of focal adhesion complexes, specialized areas of cells that promote cell adhesion (144). Associated with focal adhesion complexes in the actin cytoskeleton, the 125-kDa protein, FAK, is highly expressed in the arterial media and in cultured VSMCs and mediates cell adhesion (150) (Fig. 3). ANG II rapidly induces tyrosine phosphorylation of FAK to allow for cell adhesion to the extracellular matrix, and to enable activation of cytoskeletal proteins, including p130Cas, Pyk2 (41), paxillin (104), and talin (160), all of which interact to regulate cell shape and movement. p130Cas is an adaptor molecule whose proline-rich sequences and SH3 domain allow for an interaction with Pyk2 in presence of ANG II (154). It has also been shown that the interaction between p130Cas, Pyk2, and PI3K activates ribosomal p70S6 kinase, implicated in ANG II-mediated protein synthesis.

Receptor Tyrosine Kinases

PDGF receptor pathway. Migration and proliferation of VSMCs is critical in the pathogenesis of vascular disease, and platelet-derived growth factor (PDGF) is a potent stimulus for VSMC migration and proliferation (18, 100, 106, 223). Similar to other cell membrane tyrosine kinase receptors (EGFRs and insulin receptors), the PDGF receptor has an intrinsic tyrosine kinase activity. Both PDGF and ANG II activate several common pathways (such as MAPK and PLC) that are implicated in VSMC hypertrophy. ANG II has been shown to transduce growth-related signaling, independent of PDGF, via the PDGF receptor. It has been shown that ANG II stimulates PDGF-β-receptor phosphorylation via Shc, independent of calcium (68). Linesman and colleagues (112) showed that stimulation of rat aortic smooth muscle cells by ANG II induced the formation of a Shc and Grb2 complex with the PDGF receptor independent of PDGF, a response blocked by losartan. Other evidence shows that ANG II induces tyrosine phosphorylation of PDGF-β-receptor and increases ERK activity of rat VSMCs in vitro (112). In stroke-prone spontaneously hypertensive rats, treatment with ACE-I reduced aortic PDGF-β-receptor phosphorylation and ERK activity (96), implicating PDGF as downstream of RAS in hypertensive vascular remodeling.

Epidermal growth factor receptor pathway (EGFR). Trans-activation of EGFR is a major mechanism by which ANG II influences growth-related signaling pathways. ANG II-mediated EGFR activation occurs in the cholesterol-rich domains of caveolae in a Src-dependent and redox-sensitive manner (58, 203). Studies show that this activation is also dependent on intact microtubules and occurs via calcium-dependent and -independent pathways (43, 203). These pathways lead to activation of a disintegrin and metalloproteinase (ADAMs), causing release of heparin-binding EGF (HB-EGF) (5, 19). Recently, in COS-7 cells, ANG II-activated ADAM-17 has...
been identified as the metalloproteinase that promotes HB-EGF shedding (123, 140). HB-EGF induces conformational changes in EGFRs, allowing them to dimerize and autophosphorylate on tyrosine (151). Once activated, EGFRs serve as a docking site for Grb2/Shc/Sos complexes, inducing two major transduction pathways: the PI3K/PDK1/Akt cascade, which leads to cellular metabolism, growth, survival, and remodeling, and the Ras/Raf/ERK pathway which leads to cell growth, hypertrophy, and inflammation. Kagiyama et al. (87) reported that EGFR activation is required for ANG II-mediated hypertension and left ventricular hypertrophy, both of which are attenuated when rats are treated with an intravenous infusion of antisense oligodeoxynucleotide to EGFR. These studies and many others point to EGFR as an important factor in growth and hypertrophy caused by ANG II.

**Insulin Receptor Signaling Pathway.** In addition to direct activation of signaling pathways, ANG II influences signal transduction mechanisms induced by other agonists as well. A prime example of this is insulin signaling. In vivo studies in rats show that infusion of ANG II induces insulin resistance (139, 148); patients with an imbalance in RAS homeostasis exhibit decreased insulin sensitivity (99, 138). Further proof of this observation is that pharmacological blockade of the RAS with ACE-I and ARBs improves insulin resistance and diabetic complications (69, 77).

The insulin receptor’s ability to autophosphorylate and phosphorylate other substances results in activation of pathways that lead to insulin’s metabolic, transcriptional, and mitogenic effects (146). Once activated, the insulin receptor induces tyrosine phosphorylation of insulin receptor substrate (IRS-1), which enables its interaction with p85 (regulatory subunit of PI3K), activating the PI3K pathway, its downstream effectors PDK1 and Akt, and ultimately glucose transport. Serine phosphorylation inactivates IRS-1 both by uncoupling it from downstream effectors and by targeting it for degradation in the proteasomal pathway (129, 208). Even though as many as 35 potential serine/threonine phosphorylation sites have been identified on IRS-1, murine Ser^{307} (human Ser^{312}) has become apparent as an important site in its proteasome-mediated degradation (59).

Follì et al. (46) showed that in rat aortic smooth muscle cells, ANG II impairs insulin-mediated IRS-1 tyrosine phosphorylation and coupling of the insulin receptor to PI3K. ANG II has also been shown to increase serine phosphorylation of the insulin receptor β-subunit, and it has a direct effect on PI3K activity by increasing serine phosphorylation of p85 (46). In addition, ANG II inhibits insulin-stimulated IRS-1 association with p85 in a dose-dependent manner. Another mechanism by which ANG II interferes with insulin signaling emerges from the fact that ANG II stimulates tyrosine phosphorylation of PDK1 on Tyr^{9} (protein interacting domain) in a Src-dependent, ROS-sensitive manner (188). In the presence of mutant PDK1, ANG II-induced serine phosphorylation of IRS-1 is reduced, inhibiting its degradation, and suggesting that PDK1 is involved in ANG II-induced insulin resistance (187). Other kinases such as PKC-α have also been shown to interfere with insulin signaling via ANG II (126). Furthermore, Andreozzi et al. (6) recently demonstrated that in human umbilical vein endothelial cells, ANG II increases phosphorylation of IRS-1 Ser^{307} (via ERK) and IRS-1 Ser^{312} (via JNK), causing impaired insulin signaling. Hypertension and diabetes often present together, indicating that interaction between ANG II and insulin signaling plays an important role in cardiovascular pathology. Interruption of IRS-1 signaling by ANG II at multiple levels may explain the severity of vascular disease seen in diabetic patients.

**PHYSIOLOGICAL EFFECTS OF ANG II**

The physiological importance of ANG II in the cardiovascular system cannot be overstated. Within seconds to minutes of binding to AT_{1}Rs, it activates signaling pathways leading to VSMC contraction, maintaining vascular tone. ANG II is extremely important in modulating minute to minute changes that occur in our spatial adaptation. For example, when we stand up from a supine position, the endocrine function of ANG II may slow atherosclerotic disease progression and stabilize vulnerable plaques, partially explaining the benefits seen with ACE-I and ARB therapy.

**CARDIOVASCULAR PATHOLOGY**

ANG II affects virtually all vascular cells (endothelial cells, smooth muscle cells, fibroblasts, monocytes/macrophages, and even cardiac myocytes), and thus, is critical in disease development (Fig. 4). Changes in the phenotype and morphology of these cells, variable gene expression, and enhanced responsiveness to stimuli lead to vascular pathology. In atherosclerotic plaques, the local RAS system is active, with high levels of ACE, ANG II, and AT_{1}R (167). Antagonism of actions of ANG II may slow atherosclerotic disease progression and stabilize vulnerable plaques, partially explaining the benefits seen with ACE-I and ARB therapy.

**ANG II and Endothelial Dysfunction**

Even though the principal targets of ANG II are VSMCs, it has multiple effects on endothelial cells (ECs), such as producing ROS, activating apoptotic signaling pathways, and promoting thrombosis. In endothelial cells, ANG II regulates the production of NO, formed by nitric oxide synthase (NOS). Exposure to ANG II increases eNOS mRNA and NO production in human endothelial cells (166). In people with enhanced RAS activity, ROS-mediated endothelial dysfunction combined with vascular growth and inflammation has been implicated in atheroma formation. The increase in oxidative stress caused by ANG II leads to impaired endothelial relaxation and endothelial dysfunction (153). The intracellular ROS have been shown to activate transcription of nuclear factor κ B (NF-κB) and stimulate degradation of its cytoplasmic inhibitor, IκB (152). NF-κB gene expression results in increased levels of VCAM-1, an important factor in endothelial cell adhesion.
This observation is concordant with the report from Arenas et al. (7), who showed that ANG II modulates the secretion of inflammatory cytokines TNF-α and matrix metalloproteinase (MMP)-2 from ECs. TNF-α is also an important contributor to vascular inflammation, and its levels are elevated in vascular disorders. Many of the effects of TNF-α are similar to effects of ANG II; indeed, ANG II has been shown to stimulate the production of TNF-α through a PKC-dependent pathway in macrophages (89).

In the vessel wall, homeostatic mechanisms balance thrombosis with fibrinolysis. Plasminogen-activator inhibitor type 1 (PAI-1) inhibits tissue plasminogen activator (t-PA) and urokinase, tipping the balance in favor of thrombosis. In VSMCs and ECs, exposure to ANG II leads to increased levels of PAI-1 mRNA (45). ANG II-mediated inhibition of fibrinolysis and its induction of cell adhesion molecules such as VCAM-1 and ICAM-1 (via NF-κB activation) provide for further mechanisms by which ANG II initiates and causes progression of atherosclerosis. In endothelial cells, ANG II has been shown to induce the LDL receptor (107), which is critical in atherosclerotic lesion formation. Thus, ANG II plays a key role in modulating endothelial function, and its enhanced presence contributes to endothelial dysfunction and inflammation.

**ANG II and Vascular Inflammation**

The role of ANG II in atherosclerosis has been well established. In apolipoprotein E-deficient mice, infusion of ANG II causes accelerated atherosclerosis and aneurysm formation (1, 211). In monocytes, macrophages, VSMCs, and endothelial cells, ANG II activates NF-κB, which induces the production of cell adhesion molecules such as VCAM-1, ICAM-1, and E-selectin, and chemokines such as monocyte chemoattractant protein (MCP-1), IL-6, and IL-8 (157, 158, 167). In VSMCs, the induction of MCP-1 and IL-6 by ANG II is dependent on the activation of NAD(P)H oxidase (27, 97, 121).

Cytokines have been shown to play a major part in development and progression of atherosclerotic lesion formation. For example, human atherosclerotic plaques express elevated levels of the inflammatory cytokine interleukin-18 (IL-18) compared with normal arterial tissue (54). Recently, in a series of experiments, Sahar et al. (162) demonstrated that IL-18 activates Src, PKC, and MAPK. In ANG II-stimulated VSMCs, the effects of IL-18 were enhanced via activation of NF-κB; ANG II also induced mRNA expression of IL-18 receptors via STAT 3. The cross-talk with IL-18 signaling pathways may prove to be one of the mechanisms by which ANG II mediates its local proatherogenic effects in VSMCs.

**ANG II and Vascular Hypertrophy and Remodeling**

Over the past decade, in vitro and in vivo experiments have shown that ANG II is an important growth factor, causing cell proliferation, VSMC hypertrophy, cell differentiation, and apoptosis (38). Depending on the cell type and cytokine milieu, ANG II appears to have different growth effects (proliferation vs. hypertrophy). These differential growth effects are in part regulated by p27kip1, a cyclin-dependent kinase (CDK) inhibitor; CDKs are suppressed in presence of high levels of p27kip1, preventing cells from progressing in the cell cycle. Braun-Dullaeus et al. (22) showed that in ANG II-treated VSMCs, CDK2 activity was suppressed (secondary to failure of p27kip1 repression), leading to G1-phase arrest and cell hypertrophy.

Another mechanism implicating ANG II in cell growth comes from the observation that elevation in blood pressure affects cell growth. In rats, ANG II infusion for 2 wk. leads to hypertension and VSMC hypertrophy (115). Shear stress from elevated blood pressure has been shown to upregulate ANG II receptors (157), linking hypertension to vascular remodeling. ANG II also stimulates the production of MMPs, which are necessary for vascular remodeling (39).

Many studies support the observation that ANG II also has direct effects on myocardial cells, including hypertrophy (36, 113, 149). These effects are known to be mediated by AT1Rs; in vivo experiments in rats show that AT1R antagonists prevent ANG II-induced cardiac hypertrophy (95). The AT1R also has been shown to play a role in neointima formation via prolif-
eration of VSMCs after balloon injury (93). Kim et al. (95) showed that in rat arteries, blockade of ANG II inhibits activation of ERK/MAPKs, which are implicated in apoptosis and cell proliferation.

**ANG II and Extracellular Matrix**

In the pathogenesis of atherosclerosis and restenosis, cellular deposition of extracellular matrix (ECM) is an important component in VSMC migration and adhesion. Accumulation of ECM and reduced ECM turnover play a role in the development of vascular restenosis, hypertrophy, and heart failure after an ischemic insult to the myocardium. ANG II has been implicated in synthesis of the extracellular matrix protein collagen via both AT1Rs and AT2Rs (90, 124). ANG II-induced EGFR- and MAPK-dependent pathways may participate in matrix formation and regulation (122, 193). Indeed, ACE inhibition has been shown to limit cardiac remodeling. Fibroblast-derived ANG II exerts its local paracrine effects by stimulating the production of collagen (86). In atherosclerotic lesions, abnormal accumulation of proteoglycans has been noted (44, 79). In hypertensive rats, AT1R antagonists cause proteoglycan changes that control cell adhesion, migration, and differentiation (79, 165). Shimizu-Hirota et al. (176) showed that the ANG II-induced increase in proteoglycan synthesis was attenuated by the EGFR inhibitor AG1478 and by the MEK inhibitor PD98059. Besides regulating structural components such as collagen, ANG II has also been implicated in adhesive remodeling. Earlier, Moriguchi et al. (125) reported that ANG II-mediated EGFR transactivation regulates fibronectin and TGF-β synthesis. Furthermore, production of matrix metalloproteinases (like MMP-2) and breakdown of collagen IV is also modulated by ANG II (110). Thus, ANG II acts on several different components of ECM formation and deposition to influence matrix turnover, and many of the mechanisms and pathways that integrate ECM formation and deposition with ANG II signaling are still being discovered.

**ANG II AND VASCULAR DISEASE**

Pathologic ANG II-induced signaling in vascular, endothelial, and cardiac cells promotes ROS production, inflammation, platelet activation, altered vasoreactivity, growth, migration, and fibrosis, all of which combine to ultimately cause diseases such as hypertension, atherosclerosis, restenosis, heart failure, chronic kidney disease, insulin resistance, and tumor progression. Improved clinical outcomes after treatment with ACE-Is and ARBs confirms the importance of ANG II in the pathogenesis of these diseases (51, 77, 220). ANG II may also provide a link between atherosclerotic risk factors such as hypercholesterolemia and hypertension, since high cholesterol levels have recently been shown to increase angiotensinogen and angiotensin (34). In apolipoprotein E-deficient mice, inhibition of AT1Rs by losartan (an ARB) prevents lipid peroxidation, decreasing atherosclerotic lesion formation (91). Conversely, ANG II infusion increases aortic atherosclerosis and aneurysm formation, independent of blood pressure (33). Male apoE/AT1AR double knockout mice also have reduced atherosclerosis, indicating that alterations in AT1R expression affect vascular pathology (210). Furthermore, Yang et al. (218) have shown that in hypercholesterolemic rabbits, AT1R expression is increased, which results in altered ANG II-induced vasoreactivity and may lead to pathology. Consistent with this is the finding that after neointimal injury, ANG II receptor expression is increased (209).

Recently, other beneficial effects of ACE-Is and ARBs have been discovered. Interestingly, peroxisome proliferator-activated receptor (PPAR) agonists reduce ANG II-induced oxidative stress, inflammation, and hypertension (35). Telmisartan (an ARB) modulates PPAR-γ, a therapeutic target of diabetic drugs (15). Another PPAR-γ agonist, rosiglitazone, lowers blood pressure and improves vascular function in transgenic hypertensive mice that express human renin and angiotensinogen genes (159). These recent developments provide insight into the mechanisms by which the RAS and AT1Rs interact with other systems to influence cardiovascular pathology.

**CONCLUSIONS AND FUTURE DIRECTIONS**

The pathogenesis of chronic vascular diseases is dependent on cross-talk of various cell signaling systems. In VSMCs and ECs, interactions between lipoproteins, the RAS, and insulin is key to understanding the development and progression of vascular diseases, including hypertension, diabetes, myocardial infarction, stroke, transplant vasculopathy, and in-stent restenosis. In recent years, ANG II has been shown to generate oxidative stress in vessel wall, and has proved to be a major stimulator of inflammation, thrombosis, and fibrosis. The specificities of the signal transduction pathways mediated by ANG II remain under intense investigation, and the many downstream effectors of AT1Rs pose exciting therapeutic venues. However, most of the experiments have involved in vitro studies, and discovering the mechanisms of these pathways in vivo has posed a challenge. Novel pharmacological approaches such as virus-mediated gene delivery to block various components of the RAS and study the subsequent effects are exciting. Transgenic and knockin/knockout rodents have revolutionized our efforts to discover the mechanisms of ANG II signaling, and other animal models will provide even a greater insight into the workings of the RAS. Much work yet remains to be accomplished to advance our knowledge of the RAS in the pathogenesis of cardiovascular disease and to develop therapeutic strategies to ultimately affect morbidity and mortality.

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