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Recent advances in angiotensin II signaling

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Abstract

Angiotensin II (Ang II)* is a multifunctional hormone that influences the function of cardiovascular cells through a complex series of intracellular signaling events initiated by the interaction of Ang II with AT_1 and AT_2 receptors. AT_1 receptor activation leads to cell growth, vascular contraction, inflammatory responses and salt and water retention, whereas AT_2 receptors induce apoptosis, vasodilation and natriuresis. These effects are mediated via complex, interacting signaling pathways involving stimulation of PLC and Ca^{2+} mobilization; activation of PLD, PLA_2, PKC, MAP kinases and NAD(P)H oxidase, and stimulation of gene transcription. In addition, Ang II activates many intracellular tyrosine kinases that play a role in growth signaling and inflammation, such as Src, Pyk2, p130Cas, FAK and JAK/STAT. These events may be direct or indirect via transactivation of tyrosine kinase receptors, including PDGFR, EGFR and IGFR. Ang II induces a multitude of actions in various tissues, and the signaling events following occupancy and activation of Ang receptors are tightly controlled and extremely complex. Alterations of these highly regulated signaling pathways may be pivotal in structural and functional abnormalities that underlie pathological processes in cardiovascular diseases such as cardiac hypertrophy, hypertension and atherosclerosis.

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Key words
- Angiotensin receptors
- Second messengers
- Phospholipase
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- Reactive oxygen species

***Abbreviations:**

Akt/PKB, protein Ser/Thr kinase/protein kinase B
Ang II, angiotensin II
EGFR, epidermal growth factor receptor
ERK, extracellular signal-regulated kinase
FAK, focal adhesion kinase
IGFR, insulin growth factor receptor
IP_3, inositol triphosphate
JAK, Janus kinase
JNK, c-Jun N-terminal protein kinase
MAPK, mitogen-activated protein kinase
MEK, MAPK/ERK kinase
MKP-1, MAPK phosphatase 1
PDGF(R), platelet-derived growth factor (receptor)
PG, prostaglandin
PI3K, phosphatidylinositol 3-kinase
PKC, protein kinase C
PLA, phospholipase A
PLC, phospholipase C
PLD, phospholipase D
RTK, receptor tyrosine kinase
SAPK, stress-activated protein kinases
STAT, signal transducers and activators of transcription
VSMC, vascular smooth muscle cells

Introduction

Angiotensin II (Ang II) regulates blood pressure, plasma volume, sympathetic nervous activity and thirst responses. It also plays an important pathophysiological role in cardiovascular disease, including cardiac hypertrophy, myocardial infarction, hypertension and atherosclerosis. Ang II is produced systemically via the classical renin-angiotensin system and locally via the tissue renin-angiotensin system (1). Ang II was initially described as being primarily a vasoconstrictor peptide. However, recent studies demonstrate that Ang II has growth factor and cytokine-like properties as well. At the cellular level Ang II modulates contraction, it regulates cell growth, apoptosis and differentiation, it influences cell migration and extracellular matrix deposition, it is proinflammatory, it stimulates production of other growth factors (e.g., platelet-derived growth factor, PDGF) and vasoconstrictors (e.g., ET-1), and it transactivates growth factor receptors (e.g., PDGFR, epidermal growth factor receptor (EGFR), and insulin-like growth factor receptor (IGFR)) (2). The multiple actions of Ang II are mediated via specific, highly complex intracellular signaling pathways that are stimulated following initial binding of the peptide to its specific receptors (3). In mammalian cells Ang II binds to two distinct high-affinity plasma membrane receptors, AT_1 and AT_2. The term "intracellular signaling pathway" includes the complex interrelated molecular cascades that transmit information from the membrane receptor to the intracellular proteins that regulate cell activities. The present review focuses on recent advances in Ang II signal transduction in cardiovascular cells. The growth factor and cytokine-like properties of this vasoconstrictor peptide will be highlighted and implications in cardiovascular disease will be discussed.

Angiotensin receptors

AT_1 and AT_2 receptors, which are both seven transmembrane spanning G protein-coupled receptors, have been cloned and pharmacologically characterized. Pharmacologically the receptors can be distinguished according to inhibition by specific antagonists. AT_1 receptors are selectively antagonized by biphenylimidazoles such as losartan, whereas tetrahydroimidazopyridines such as PD123319 specifically inhibit AT_2 receptors (3,4). Two other Ang receptors have been described, AT_3 and AT_4 (5). However, the pharmacology of these receptors has not been fully characterized, and therefore these receptors are not yet included in a definitive classification of mammalian Ang receptors (6).

AT_1 receptor

In humans there is only a single AT_1 receptor type, whereas in rodents, two subtypes of the AT_1 receptor have been identified (AT_{1a} and AT_{1b}). To date, AT_1 receptors have been shown to mediate most of the physiological actions of Ang II and this subtype is predominant in the control of Ang II-induced vascular functions (1). In the vasculature, AT_1 receptors are expressed mainly in smooth muscle cells (7). In the heart, AT_1 receptors are present in cardiomyocytes and fibroblasts (7). Ligand-receptor binding leads to activation of G proteins through exchange of GTP for GDP, resulting in the release of α and $\beta\gamma$ complexes, which mediate downstream actions. AT_1 receptors interact with various heterotrimeric G proteins including Gq/11, Gi, Gα12 and Gα13. The different G protein isoforms couple to distinct signaling cascades. For example, Gq activation results in activation of phospholipase C (PLC), whereas GαI leads to cGMP formation. Although G protein-coupled receptors do not contain intrinsic kinase activity, they are phosphorylated on serine and threonine residues by members of the G protein receptor kinase family. AT_1 receptors are phosphorylated in the basal state and in response to Ang II stimulation. Various tyrosine kinases, including Janus kinases (JAK and TYK), Src

family kinases, and focal adhesion kinase (FAK) can tyrosine phosphorylate AT_1 receptors (8).

AT_2 receptor

The second major isoform of the Ang receptor, AT_2, is normally expressed at high levels in fetal tissues, and decreases rapidly after birth (9). In adults, AT_2 receptor expression is detectable in the pancreas, heart, kidney, adrenals, myometrium, ovary, brain and vasculature (9). In blood vessels, AT_2 receptors predominate in the adventitia and are detectable in the media. The AT_2 receptor is re-expressed in adults after vascular and cardiac injury and during wound healing and renal obstruction, suggesting a role for this receptor type in tissue remodeling, growth and/or development. The functional roles of AT_2 receptors are unclear, but these receptors may antagonize, under physiological conditions, AT_1-mediated effects by inhibiting cell growth, and by inducing apoptosis and vasodilation (10,11). Recently, *in vivo* studies of forearm blood flow in healthy human subjects found that the AT_2 receptor was not involved in modulating vascular tone in these subjects (12). Other studies suggest that AT_2 receptors also contribute to pathological processes associated with cardiac hypertrophy and inflammation (13). Signaling pathways through which AT_2 receptors mediate cardiovascular actions have recently been elucidated. Four major cascades are involved including 1) activation of protein phosphatases and protein dephosphorylation, 2) regulation of the nitric oxide-cGMP system, 3) stimulation of PLA_2 and release of arachidonic acid, and 4) sphingolipid-derived ceramide. These pathways have been reviewed elsewhere (14) and will not be discussed further here.

Intracellular signals induced by AT_1 receptors

Ang II promotes its effects by acting directly through Ang II receptors, indirectly through the release of other factors, and via cross-talk with intracellular signaling pathways of other vasoactive agents, growth factors and cytokines. AT_1 receptors are coupled to multiple, specific signaling cascades, leading to diverse biological actions. The signaling processes are multiphasic with distinct temporal characteristics.

AT_1 signaling through phospholipids (Figure 1)

Phospholipase C. One of the earliest detectable events resulting from Ang II stimulation is a rapid, PLC-dependent hydrolysis of phosphatidylinositol-4,5-bisphosphate (15). The PLC family includes three related enzymes: PLC-ß, PLC-γ, and PLC-δ which are regulated by either G proteins α and ßγ (PLC-ß), by tyrosine phosphorylation (PLC-γ) or by Ca^{2+} (PLC-δ). Classically, AT_1 receptor activation results in a rapid production of 1,4,5-inositol triphosphate (IP_3), and a more sustained release of diacylglycerol, which are involved in Ca^{2+} mobilization from

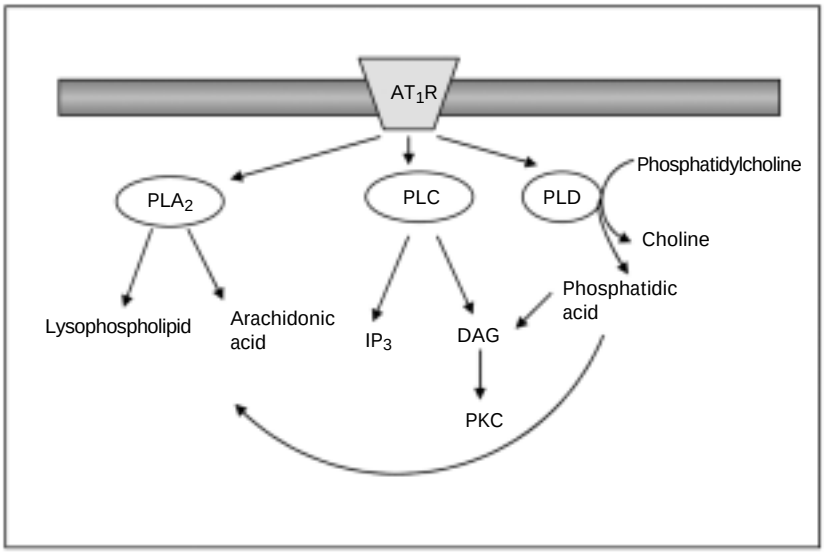

Figure 1. Phospholipid-derived second messengers resulting from Ang II-activated phospholipases. Shown are the major phospholipid-derived products from reactions catalyzed by phospholipase A (PLA), phospholipase C (PLC) and phospholipase D (PLD). AT_1R = angiotensin 1 receptor, IP_3 = inositol triphosphate, DAG = diacylglycerol, PKC = protein kinase C.

the sarcoplasmic reticulum and stimulation of protein kinase C (PKC) (16), respectively. Ang II-stimulated IP_3 generation may also be mediated, in part, via tyrosine kinase-dependent pathways. Increased intracellular Ca^{2+} results in vascular smooth muscle cell (VSMC) contraction, whereas PKC activation regulates intracellular pH through the Na^+/H^+ exchanger (15,16). PLC activation correlates temporally with initiation of contraction in isolated VSMC, as well as in intact small resistance arteries, and most likely constitutes the early signaling pathway for initiation of the Ca^{2+}-dependent, calmodulin-activated phosphorylation of the myosin light chain which leads to cellular contraction. The cellular processes underlying Ang II-induced rise in $[Ca^{2+}]_i$ and pHi following AT_1 receptor stimulation have been extensively reviewed elsewhere (15,17).

Phospholipase D. Unlike PLC, which preferentially acts on phospholipids containing phosphoinositol, PLD hydrolyzes phosphatidylcholine. The sustained activation of PLD is a major source of prolonged second messenger generation in VSMC and cardiomyocytes (18). Hydrolysis of phosphatidylcholine by PLD leads to the production of phosphatidic acid and subsequent generation of diacylglycerol by phosphatidic acid phosphohydrolase (18). Diacylglycerol is the physiological activator of PKC and is also a source of arachidonic acid. A number of PKC isoforms have been described including α, ß, δ, ε, μ, ς, and λ. Molecular mechanisms coupling AT_1 receptors to PLD involve Gßγ and their associated Gα12 subunits, Src and RhoA (19). The downstream pathways associated with Ang II-induced activation of PLD in VSMC are PKC independent, but involve intracellular Ca^{2+} mobilization and Ca^{2+} influx that is tyrosine kinase dependent. Ang II-induced PLD signaling has been implicated in cardiac hypertrophy, VSMC proliferation, and vascular contractility (20,21). These actions are mediated via phosphatidic acid and other PLD metabolites, that influence vascular generation of superoxide anions by stimulating NAD(P)H oxidase, which activate tyrosine kinases and Raf and modulate intracellular Ca^{2+} signaling (18). The long-term signaling events associated with Ang II-stimulated growth and remodeling in the cardiovascular system are dependent, in large part, on PLD-mediated responses.

Phospholipase A_2. Ang II induces activation of PLA_2, which is responsible for release of arachidonic acid from cell membrane phospholipids (22). Released arachidonic acid is metabolized by cyclooxygenases, lipoxygenases or cytochrome P450 oxygenases to many different eicosanoids in vascular and renal tissues. Cyclooxygenases catalyze the formation of prostaglandin (PG) PGH_2, subsequently converted to thromboxane by thromboxane synthase, to PGI_2 (or prostacyclin) by prostacyclin synthase, or to PGE_2, PGD_2 or $PGF_{2\alpha}$, by different enzymes (22). Lipoxygenases catalyze the formation of 5-, 12-, or 15-HPETEs, that then undergo spontaneous or peroxidase-catalyzed reduction to the corresponding HETEs and, in the case of 5-HPETE, to leukotrienes (22). Cytochrome P450 oxygenases catalyze arachidonic acid epoxidation to epoxyeicosatrieenoic acids, ω and ω-1 hydroxylation to 20- and 19-HETE, and allylic oxidation to other HETEs.

PLA_2-derived eicosanoids influence vascular and renal mechanisms important in blood pressure regulation. In VSMC and endothelial cells, these effects are mediated via AT_1 receptors, whereas in neonatal rat cardiac myocytes, neuronal cells and renal proximal tubule epithelial cells, Ang II-induced activation of PLA_2 occurs via AT_2 receptors (23). Ang II-elicited activation of vascular PLA_2 is dependent on $[Ca^{2+}]_i$, Ca^{2+}-calmodulin-dependent protein kinase II and mitogen-activated protein kinases (MAPK) (22,23). Activated PLA_2 and its metabolites in turn activate Ras/MAPK-dependent signaling pathways, amplifying PLA_2 activity and releasing additional arachidonic acid by

a positive feedback mechanism. Ang II-generated eicosanoids regulate vascular contraction and growth, possibly by activating MAPK and redox-sensitive pathways. Thromboxanes are involved in Ang II-stimulated contraction, whereas vasorelaxant PGs such as PGE_2 and PGI_2 attenuate Ang II-mediated vasoconstriction in some vascular beds. Lipoxygenase-derived eicosanoids also influence Ang II-elicited actions in VSMC. 12-HETE facilitates the stimulatory actions of Ang II on Ca^{2+} transients in cultured cells. Lipoxygenase inhibitors attenuate the vasoconstrictor action of Ang II and decrease blood pressure in SHR (24).

AT_1-mediated tyrosine phosphorylation

A recent development in the field of Ang II signaling is the demonstration that AT_1 receptor activation is associated with increased protein tyrosine phosphorylation and activation of MAPK. These processes are characteristically associated with growth factors and cytokines. Accordingly, it is becoming increasingly evident that in addition to its potent vasoconstrictor properties, Ang II has mitogenic- and inflammatory-like characteristics. Ang II stimulates phosphorylation of many non-receptor tyrosine kinases including PLC-γ, Src family kinases, JAK (JAK and TYK), FAK, Ca^{2+}-dependent tyrosine kinases (e.g., Pyk2), p130Cas and phosphatidylinositol 3-kinase (PI3K). In addition Ang II influences activity of receptor tyrosine kinases (RTK), such as EGFR, PDGFR and IGFR. The role of tyrosine kinases in Ang II-mediated signaling has been extensively reviewed (1,2,8). Only recent developments are discussed here.

Non-receptor tyrosine kinase activation (Figure 2)

Src family kinases. To date at least 14 Src-related kinases have been identified, of which the 60-kDa c-Src is the prototype. c-Src is abundantly expressed in vascular smooth muscle and is rapidly activated by Ang II in VSMC (25). Src plays an important role in Ang II-induced phosphorylation of PLC-γ and IP_3 formation. Src, intracellular Ca^{2+} and PKC regulate Ang II-induced phosphorylation of p130Cas, a signaling molecule involved in integrin-mediated cell adhesion. Src has also been associated with Ang II-induced activation of Pyk2 and extracellular signal-regulated kinases (ERKs) as well as activation of other downstream proteins including pp120, p125Fak, paxillin, JAK2, signal transducers and activators of transcription 1 (STAT1), Gα, caveolin, and the adapter protein, Shc (26). Studies in VSMC isolated from human resistance arteries suggest that c-Src may also be important in the regulation of Ang II-stimulated

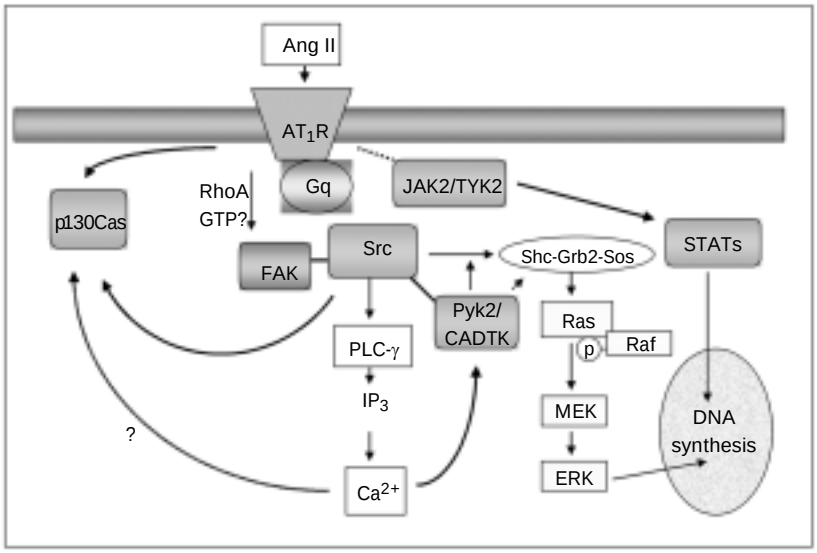

Figure 2. Tyrosine kinase pathways stimulated by angiotensin II (Ang II) in vascular smooth muscle cells. Ang II activates Src, which regulates phospholipase C-γ (PLC-γ)- and extracellular signal-regulated kinase (ERK)-dependent signaling pathways. Ang II binding to the angiotensin 1 receptor (AT_1R) induces the physical association and activation of JAK2/TYK2 (Janus kinase/s) as indicated by the dashed line. JAK2/TYK2 phosphorylates STAT proteins (signal transducers and activators of transcription) that are translocated to the nucleus where they activate gene transcription. Ang II also activates focal adhesion kinase (FAK) which possesses sites favored for phosphorylation by Src. FAK associates with paxillin and talin that associate with actin. The link between AT_1 receptor and FAK is unknown, but the Rho family of GTPases are potential candidates. Pyk2 and CADTK (Ca^{2+}-dependent tyrosine kinase) are activated by Ang II through Ca^{2+}-dependent pathways. Activated Pyk2 regulates Src and ERK-dependent signaling cascades. p130Cas is transiently activated by Ang II, possibly via a Ca^{2+}-dependent pathway. Phosphorylated p130Cas may be important in the regulation of α-actin expression. PI3K activation by Ang II leads to Akt/protein kinase B activation, which in turn stimulates cell survival pathways and activation of p70 S6-kinase.

Ca^{2+} mobilization (27). Furthermore, c-Src mediates Ang II regulation of plasminogen activity in bovine aortic endothelial cells. Activation of c-Src is required for cytoskeletal reorganization, focal adhesion formation, cell migration and cell growth (28). Increased activation of c-Src by Ang II may be an important mediator of altered VSMC function in hypertension.

Janus family kinases, *tyrosine kinase and STAT activation.* Similar to classical cytokine receptors, the AT_1 receptor stimulates JAK2 and TYK2, members of the JAK family (29). AT_1 receptor-induced activation of JAK leads to phosphorylation of the STAT proteins p91/84 (STAT1α/ß), p113 (STAT2) and p92 (STAT3), which are transcription factors. Ang II-induced tyrosine phosphorylation and nuclear translocation of STAT1 require JAK2 and p59 Fyn kinase (a member of the Src family of kinases). p59 Fyn appears to act as a docking protein for both JAK2 and STAT1, which facilitates JAK2-mediated phosphorylation of STAT1. JAK proteins are key mediators of mRNA expression and are characterized as "early growth response genes". JAK phosphorylates STAT proteins that are translocated to the nucleus, where they activate gene transcription (30). Electroporation of antibodies against STAT1 and STAT3 abolished VSMC proliferative responses to Ang II, but not to other growth factors, implicating an essential role of STAT proteins in Ang II-induced cell proliferation (30). The JAK-STAT signaling pathway activates early growth response genes, and may be a mechanism whereby Ang II influences vascular and cardiac growth, remodeling and repair.

Focal adhesion kinase and proline-rich tyrosine kinase 2. Ang II promotes cell migration and induces changes in cell shape and volume by activating FAK-dependent signaling pathways (31). Focal adhesion complexes, specialized sites of cell adhesion, act as supramolecular structures for the assembly of signal transduction mediators. The best-characterized tyrosine kinase localized to focal adhesion complexes is a 125-kDa protein, FAK. FAK exhibits extracellular matrix-dependent tyrosine autophosphorylation and physically associates with two non-RTK, c-Src and p59 Fyn (pp59), via their SH2 domains (32). FAK autophosphorylation may also result in physical associations with PI3K, which is a 'downstream' tyrosine kinase involved in trophic cellular responses. As a consequence of its association with c-Src, FAK undergoes further tyrosine phosphorylation, which results in FAK binding to Grb2, an association with the GDP-GTP exchange protein, Sos, and Ras. This in turn leads to ERK1/2 activation. FAK is abundant in developing blood vessels, and elevation of its phosphotyrosine content in VSMC is a rapid response to Ang II. Ang II-induced activation of FAK causes its translocation to sites of focal adhesion with the extracellular matrix and phosphorylation of paxillin and talin, which may be involved in the regulation of cell morphology and movement (32). AT_1-induced FAK activation also plays an important role in Ang II-mediated hypertrophic responses in VSMC. The link between the AT_1 receptor and FAK is unknown, but the Rho family of GTPases may be important.

Another FAK family member, Pyk2, also called cell adhesion kinase-ß, related adhesion focal tyrosine kinase and calcium-dependent tyrosine kinase (the rat homologue of Pyk2), is activated by AT_1 receptors and is dependent on increased intracellular Ca^{2+} (32). Since Pyk2 is a candidate to regulate c-Src and to link G protein-coupled vasoconstrictor receptors with protein tyrosine kinase-mediated contractile, migratory and growth responses, it may be a potential point of convergence between Ca^{2+}-dependent signaling pathways and protein tyrosine kinase pathways in VSMC. In endothelial cells the balance of Pyk2 tyrosine phosphorylation in response to Ang II is controlled by Yes kinase (Src family kinase) and by a tyrosine phosphatase SHP-2 (33).

p130Cas. p130Cas is an Ang II-activated tyrosine kinase that plays a role in cytoskeletal rearrangement (34). This protein serves as an adapter molecule because it contains proline-rich domains, an SH3 domain, and binding motifs for the SH2 domains of Crk and Src. p130Cas is important for integrin-mediated cell adhesion, by recruitment of cytoskeletal signaling molecules such as FAK, paxillin and tensin to the focal adhesions. In cultured VSMC, Ang II induces a transient increase in p130Cas tyrosine phosphorylation (28). Some investigators have found this phosphorylation to be dependent on Ca^{2+}, c-Src and PKC, and to require an intact cytoskeletal network (28). Other studies reported that Ang II-induced activation of p130Cas is Ca^{2+} and PKC independent (35). Although the exact functional significance of Ang II-induced activation of p130Cas is unclear, it might regulate α-actin expression, cellular proliferation, migration and cell adhesion. p130Cas also plays a critical role in cardiovascular development and actin filament assembly.

Phosphatidylinositol 3-kinase. PI3K is a heterodimeric enzyme composed of a p85 adapter and a p110 catalytic subunit. PI3K catalyzes the synthesis of 3-phosphorylated phosphoinositides. The major products of PI3K influence cell survival, metabolism, cytoskeletal reorganization and membrane trafficking and have recently been identified to play an important role in the regulation of VSMC growth (36). PI3K, characteristically associated with tyrosine kinase receptors, is also activated by AT_1 receptors (37,38). In VSMC Ang II stimulates activity, phosphorylation and migration of PI3K, and induces translocation of the p85 subunit from the perinuclear area to foci throughout the cytoplasm and the cytoskeletal apparatus (37). PI3K inhibition by wortmannin and LY294002 blocks Ang II-stimulated hyperplasia in cultured rat cells, suggesting the important regulatory role of this non-RTK in VSMC growth (37). Several molecular targets for PI3K have been identified, including centaurin, the actin-binding-protein profilin, phosphoinositide-dependent kinases, the atypical PKCs, PLC-γ, Rac1, c-Jun N-terminal protein kinase (JNK) and the protein Ser/Thr kinase (Akt)/protein kinase B (PKB) (38). Akt/PKB has recently been identified as an important PI3K downstream target in Ang II-activated VSMC. It regulates protein synthesis by activating p70 S6-kinase and it modulates Ang II-mediated Ca^{2+} responses in aortic cells by stimulating Ca^{2+} channel currents. Akt/PKB has also been implicated to protect VSMC from apoptosis and to promote cell survival by influencing Bcl-2 and c-Myc expression and by inhibiting caspases (38). Mechanisms whereby the AT_1 receptor mediates activation of PI3K-dependent Akt/PKB are unclear, but redox-sensitive pathways and c-Src may be important. Although the exact role of PI3K in Ang II signaling in VSMC has not yet been established, it is possible that this complex pathway may control the balance between mitogenesis and apoptosis.

Receptor tyrosine kinases

Increasing evidence suggests that mitogenic responses to AT_1 receptor activation may be mediated by activation of RTK. Ang II can activate RTK, even though it does not directly bind to RTK. This process of transactivation has been demonstrated for EGFR, PDGFR and IGFR and has recently been reviewed (39). Mechanisms underlying Ang II-induced transactivation of RTKs include activation of tyrosine kinases (Pyk2 and Src) and redox-sensitive processes (39). EGFR transactivation seems to be a Ca^{2+}-dependent process, whereas PDGFR transactivation is Ca^{2+} independent. Recently, a novel concept for EGFR transactivation by G protein-coupled receptors was suggested. In response to G protein-coupled receptor agonists (endothelin-1, thrombin, carbachol, lysophosphatidic acid and tetradecanoyl-

phorbol-13-acetate), heparin binding-EGF is generated by cleavage of pro-heparin-binding-EGF by metalloproteinase (40). Free heparin-binding-EGF then binds to EGFR resulting in EGFR homodimerization and autophosphorylation. Similar processes have been demonstrated for IGFR transactivation. The role of these mechanisms in RTK transactivation by Ang II is unclear as some studies failed to demonstrate a role for metalloproteinase in AT_1-mediated EGFR transactivation in VSMC and cardiac fibroblasts (39).

In vitro evidence suggests that AT_1 receptor-induced EGFR transactivation is important for some of the trophic effects of Ang II. For example, AT_1 receptor-elicited tyrosine phosphorylation and activation of EGFR stimulated downstream activation of ERK1/2 and VSMC hyperplasia (41). In rat VSMC, both Ang II-induced nuclear proto-oncogene expression and increase in c-Fos protein were prevented by treatment with EGFR kinase inhibitor. Ang II-mediated EGFR transactivation also plays a role in p70 ribosomal protein S6-kinase-induced protein synthesis (41). Furthermore, recent studies have demonstrated that EGFR activation is involved in Ang II-induced vascular contraction.

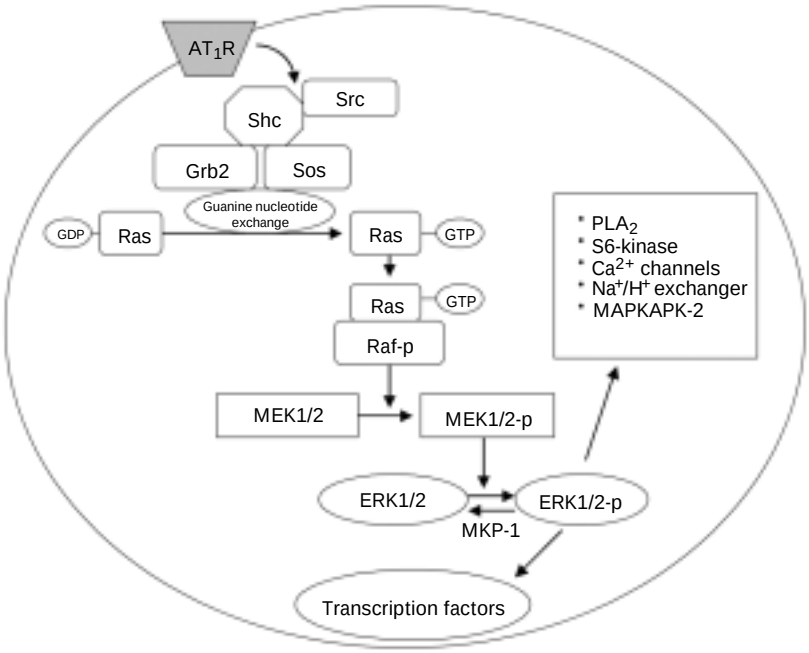

Figure 3. Ang II-stimulated extracellular signal-regulated kinase (ERK)-dependent signaling pathways in vascular smooth muscle cells. The ERK phosphorylation cascade is initiated by Ang II binding to angiotensin 1 receptors (AT_1R) that induce Shc-Grb2-Sos formation (tyrosine phosphorylation of Shc) which in turn facilitates guanine nucleotide exchange on the small G protein Ras-GDP/GTP. Activated Ras-GTP interacts with the Ser/Thr kinase Raf (mitogen-activated protein kinase (MAPK) kinase kinase (MAPKKK)) which translocates to the cell membrane. Activation of Raf leads to phosphorylation of two serine residues present in MEK (MAPK/ERK kinase) which in turn phosphorylates Thr/Tyr and activates MAPK, present as a 44- (ERK-1) and a 42-kDa isoform (ERK-2). Phosphorylated ERK has diverse intracellular protein targets which it phosphorylates and activates. Dephosphorylation of ERK is accomplished by activation of MAPK phosphatase-1 (MKP-1). PLA_2 - phospholipase A_2.

Mitogen-activated protein kinases (Figure 3)

Mitogen-activated protein kinases are a family of serine/threonine protein kinases that mediate nuclear transduction of extracellular signals by intracellular protein phosphorylation, leading to a cascade of transcription factor activation, enhanced gene expression and trophic cellular responses. Mammalian MAPKs are grouped into six major subfamilies: a) ERK1/2 (also known as p42-kDa MAPK and p44-kDa MAPK, respectively), b) JNK/stress-activated protein kinases (JNK/SAPK), c) p38 MAPK, d) ERK6, p38-like MAPK, e) ERK3, and f) ERK5 (also called Big MAPK 1) (42). MAPK-dependent signaling pathways have been associated with cellular growth and apoptosis, cellular differentiation and transformation and vascular contraction. ERK1/2 is activated in response to growth and differentiation factors, whereas JNKs and p38 MAPK are usually activated in response to inflammatory cytokines and cellular stress (42). Ang II differentially activates the three major members of the MAPK family, ERK1/2, JNKs and p38 MAPK (43,44). Induction of MAPK activation typically involves phosphorylation by a MAPK kinase, also known as MEK. MEK is, in turn, regulated by other MEK kinases, including Raf-1. Although activated by similar stimuli, the signaling processes leading to JNK and p38 MAPK activation are quite different. The best characterized MAPK cascade is the Raf-

Ras-MEK-ERK1/2 pathway.

Events downstream to MAPK activation are numerous and heterogeneous and include PLA_2, cytoskeletal proteins, the MAPK-activated protein kinase 2, and the $pp90^{rsk}$ protein kinase, which can translocate to the nucleus and activate transcription factors (42). Once phosphorylated, ERKs translocate to the nucleus to phosphorylate transcription factors and thereby regulate gene expression of cell cycle-related proteins. In VSMC, another downstream target of ERK is the serine/threonine protein kinase $pp90^{rsk}$, which phosphorylates the S6 ribosomal protein and stimulates protein synthesis. ERK1/2 activation ultimately results in enhanced proto-oncogene expression, and activation of the activator protein 1 complex transcription factor and probably regulates cell cycle progression as well as protein synthesis in VSMC. Ang II may also induce protein synthesis by an ERK-independent pathway in part via activation of the 70-kDa S6-kinase. Other downstream targets of MAPK include cyclooxygenase-2, the contractile regulatory protein h-caldesmon, the high-molecular weight form of caldesmon, myelin basic protein, microtubule-associated protein, Ca^{2+} channels and the Na^+/H^+ exchanger (42). The functional outcome of MAPK activation probably depends in part on the availability of downstream substrates.

Ang II activates the MAPK signaling cascade at various intracellular levels. It induces tyrosine and threonine phosphorylation of ERK1/2, JNK/SAPK and p38 MAPK in cultured VSMC, as well as in intact arteries. It stimulates phosphorylation of Ras, Raf and Shc and it increases activity of MEK kinase and MEK. In addition, Ang II increases activation of vascular Src and Pyk2, potential links between the Ang receptor and ERK. Ang II-stimulated ERK1/2 is associated with increased expression of the early response genes c-*fos,* c-*myc* and c-*jun*, DNA synthesis, cell growth and differentiation and cytoskeletal organization (42).

In addition to ERKs, Ang II activates JNK/SAPKs, which regulate VSMC growth by promoting apoptosis or by inhibiting growth (45). Ang II induces phosphorylation of JNK/SAPK via p21-activated kinase (αPAK) which is dependent on intracellular Ca^{2+} mobilization and on PKC activation. Following phosphorylation, the isoforms JNK-1 and JNK-2 translocate to the nucleus to activate transcription factors, such as c-Jun, ATF-2 and Elk-1 (46). Ang II appears to activate VSMC ERK1/2 and JNK/SAPK via different signaling pathways. ERK phosphorylation occurs via a Ca^{2+}-dependent or -independent pathway that involves c-Src and the atypical PKC isoform PKC-ξ, whereas JNK/SAPK activation occurs via a Ca^{2+}-dependent pathway that involves a tyrosine kinase other than Src and a novel PKC isoform (47). Furthermore, whereas Ang II-induced phosphorylation peaks within 5 min, kinase activation is maximal at about 30 min. The functional effects of Ang II-induced signaling of ERK1/2 and JNK/SAPK in VSMC probably relate to regulation of cell growth. Ang II-activated ERK1/2 and JNK/SAPKs have opposite growth effects, with ERK1/2 being facilitative and JNK/SAPK inhibitory. These signaling processes and associated cellular functions are important in vascular damage associated with cardiovascular disease.

Ang II also phosphorylates vascular p38 MAPK, which plays an important role in inflammatory responses, apoptosis and inhibition of cell growth (48). The p38 MAPK pathway has been implicated in various pathological conditions including cardiac ischemia, ischemia/reperfusion injury, cardiac hypertrophy, progression of atherosclerosis and arterial remodeling in hypertension. The specific upstream and downstream regulators of Ang II-activated p38 in VSMC are unclear, but p38 MAPK could be a negative regulator of ERK1/2 (48). p38 MAPK has been implicated to be an essential component of the redox-sensitive signaling path-

ways in Ang II-activated VSMC (48).

Inactivation of Ang II-stimulated MAPK occurs via MAPK phosphatase 1 (MKP-1)-induced dephosphorylation of both tyrosine and threonine on MAPK. Inhibition of MKP-1 results in sustained activation of MAPK in response to Ang II, suggesting that this enzyme is primarily responsible for the termination of the MAPK signal (49). Ang II induces activation of MKP-1, as well as tyrosine phosphatase (PTP-1C), and Ser/Thr phosphatase PP2A (49). These effects appear to be mediated via the AT_2 receptor subtype, which has been associated with inhibition of cell growth and apoptosis. Accordingly, AT_1 receptors induce growth via stimulation of ERK-dependent signaling pathways, whereas AT_2 receptors oppose these effects by stimulating MKP-1 activity to inhibit ERK activity, and to arrest the cell growth signal. Termination of Ang II-stimulated MAPK activity may also involve activation of PKA, which inhibits the phosphorylation of Raf-1.

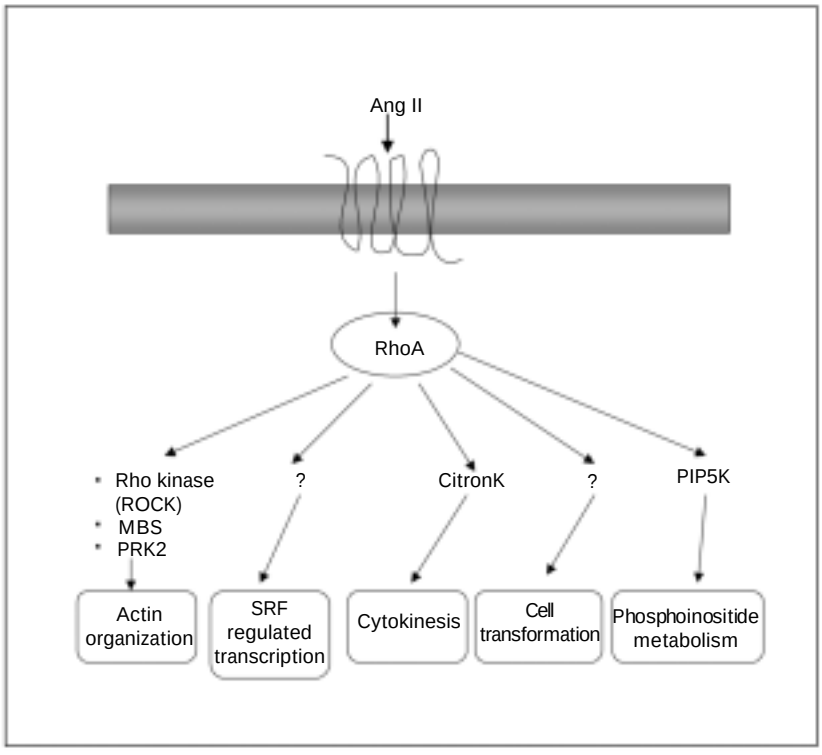

Figure 4. Rho-regulated signaling pathways by angiotensin II (Ang II). Rho is activated through AT_1 receptors. Rho regulates the activity of many signaling pathways that influence contraction, growth and cytoskeletal organization. MBS, myosin binding subunit of the myosin light-chain phosphatase; PRK2, protein kinase C-related kinase; SRF, serum response factor.

Small G proteins (Rho family) (Figure 4)

In addition to signaling through heterotrimeric G proteins, recent evidence suggests that AT_1 receptors activate monomeric small (21 kDa) guanine nucleotide-binding proteins (small G proteins) in VSMC. The small G protein superfamily comprises five subfamilies (Ras, Rho, ADP ribosylation factors, Rab and Ran) that act as molecular switches to regulate cellular responses (50). Of these, the Rho subfamily (RhoA, Rac1 and Cdc42) has been associated with Ang II signaling. In its GDP-bound form, RhoA is inactive and is mainly cytoplasmic. RhoA is activated upon the exchange of bound GDP for GTP and for its activation, requires post-translational modification (geranylgeranylation) (50). One of the major downstream targets of RhoA is Rho kinase, which promotes VSMC contraction via the phosphorylation of the myosin-binding subunit of myosin light chain phosphatase, thereby inhibiting phosphatase activity (51). This contributes to increased Ca^{2+} sensitization. Various vasoactive agonists signaling through G protein-coupled receptors enhance the sensitivity of the contractile machinery to changes in $[Ca^{2+}]_i$ via RhoA/Rho kinase. Whether this system contributes to contraction following AT_1 receptor activation awaits clarification. However, RhoA seems to play a role in AT_1-stimulated growth signaling pathways and in processes associated with atherosclerosis. Yamakawa et al. (52) demonstrated in rat VSMC that Ang II increases c-fos expression and protein synthesis via RhoA/Rho kinase-dependent mechanisms that do not involve ERK1/2 or p70 S6-kinase activation. Funakoshi et al. (53) reported that Rho kinase mediates Ang II-induced monocyte chemoattractant protein-1 expres-

sion in rat VSMC. RhoA has also been shown to be important in AT_1-mediated PLD activation.

Ang II also activates Rac1, another small G protein. Rac1 participates in cytoskeletal organization, cell growth, inflammation and regulation of NAD(P)H oxidase (50). In VSMC, Ang II activates Rac1, which is an upstream regulator of p21-activated kinase and JNK. Rac1 also plays a role in Ang II-induction of gene transcription and in the regulation of NAD(P)H oxidase, which mediates generation of superoxide anions ($\cdot O_2^-$) in vascular cells (50).

Generation of reactive oxygen species (Figure 5)

Reactive oxygen species such as $\cdot O_2^-$ and hydrogen peroxide (H_2O_2) act as intercellular and intracellular second messengers that may play a physiological role in vascular tone and cell growth, and a pathophysiological role in inflammation, ischemia-reperfusion, hypertension and atherosclerosis. Xanthine oxidase, mitochondrial oxidases and arachidonic acid are the major sources of oxidative molecules in non-vascular tissue, whereas a nonmitochondrial, membrane-associated NAD(P)H oxidase appears to be the most important source of $\cdot O_2^-$ in vascular cells (54). This enzyme transfers electrons from NADH or NADPH to molecular oxygen, producing $\cdot O_2^-$. The complete molecular structure of the vascular oxidase is unknown, but it shares some features with the neutrophil oxidase, which comprises five subunits: a 22-kDa α-subunit ($p22^{phox}$), a glycosylated 91-kDa ß-subunit ($gp91^{phox}$), which together make up cytochrome b_{558}, the electron transfer element; cytosolic components $p47^{phox}$ and $p67^{phox}$, and a low-molecular weight G protein, Rac1 or Rac2. Recent studies by Rueckschloss et al. (55) demonstrated complete homology of the cDNA sequence of $gp91^{phox}$ in human umbilical vein endothelial cells and neutrophils. Alternatively, differences in the levels of gene expression of $gp91^{phox}$ and $p67^{phox}$ were observed, with human umbilical vein endothelial cells exhibiting 1.1 and 2.5%, respectively, of the levels of expression of these genes, compared with those of neutrophils. By contrast, gene expression for $p22^{phox}$ and $p67^{phox}$ was comparable between these two cell types. These observations suggest that the levels of expression of $gp91^{phox}$ and $p67^{phox}$ genes may be rate-limiting factors for human vascular cell $\cdot O_2^-$ production. Upon activation, the $p47^{phox}$ and $p67^{phox}$ proteins are translocated to the membrane and associate with the cytochrome b_{558}, creating the active oxidase. In VSMC, Ang II increases $\cdot O_2^-$ production by activating NAD(P)H oxidase. This effect is sustained and probably contributes to long-term signaling events such as cell growth.

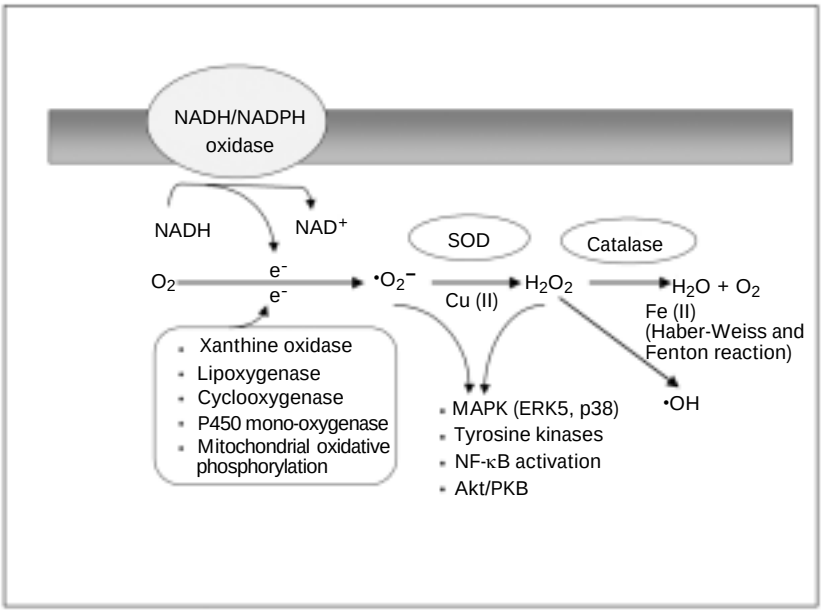

Figure 5. Generation of reactive oxygen species in the vasculature. Many enzyme systems stimulate production of superoxide anion ($\cdot O_2^-$) from O_2. These include NADH/NADPH oxidase, xanthine oxidase, lipoxygenase, cyclooxygenase, P450 mono-oxygenase and mitochondrial oxidative phosphorylation. NADH/NADPH oxidase is a multisubunit enzyme that is the major regulated source of reactive oxygen species in endothelial and vascular smooth muscle cells. Dismutation of $\cdot O_2^-$ spontaneously or enzymatically by superoxide dismutase (SOD) produces hydrogen peroxide (H_2O_2) that can undergo further reactions to generate the highly reactive hydroxyl radical ($\cdot OH$). H_2O_2 may be metabolized by catalase or peroxidases to H_2O and O_2. Downstream targets of $\cdot O_2^-$ and H_2O_2 include ERK5, p38, tyrosine kinases and NF-κB. MAPK = mitogen-activated protein kinase; ERK = extracellular signal-regulated kinase; Akt/PKB = protein Ser/Thr kinase/protein kinase B.

Generation of reactive oxygen species is regulated by various cytokines and growth factors, including Ang II, which increases $\cdot O_2^-$ and H_2O_2 production in cardiac, vascular smooth muscle, endothelial, adventitial and mesangial cells (21,54,56). Increased production of reactive oxygen species has been implicated in the pathogenesis of Ang II-induced but not catecholamine-induced hypertension (57). Mechanisms underlying oxidative stress-induced hypertension may be associated with the vascular mitogenic effects of $\cdot O_2^-$ and H_2O_2, decreased bioavailability of endothelium-derived nitric oxide and contractile actions of $\cdot O_2^-$ and H_2O_2. Growth of VSMC by Ang II has an essential redox-sensitive component, which appears to be mediated in part via activation of p38 MAPK and JNK (48). Another redox-sensitive process cascade whereby Ang II influences cell function is through phosphorylation of the cell survival protein kinase Akt/PKB and activation of NF-κB, important in inflammatory responses (54).

Ang II-induced expression of proto-oncogenes and growth factors

Long-term control of Ang II-regulated cellular growth, adhesion, migration, fibrosis and collagen deposition within the vasculature involves protein synthesis. Ang II induces the expression of several proto-oncogenes in human and rat VSMC, including c-*fos*, c-*jun*, c-*myc*, *erg-1*, VL-30, and proto-oncogene/activator protein 1 complex (58). Stimulation of early response genes by Ang II is associated with increased gene expression and production of growth factors, such as PDGF, EGF, transforming growth factor-ß, IGF-1, basic fibroblast growth factor and platelet activating factor, vasoconstrictor agents, such as ET-1, adhesion molecules such as ICAM-1, VCAM-1 and E-selectin, and integrins αvß3 and ß5, and chemotactic factors such as tumor necrosis factor-α and monocyte chemoattractant protein-1 (58). These agents may contribute indirectly to the trophic and inflammatory actions of Ang II in cardiovascular tissue.

Ang II influences the architecture and integrity of the vascular wall by modulating cell growth as well as regulating extracellular matrix composition. Ang II increases expression and production of fibronectin, collagen type 1, tenascin, glycosaminoglycans, chondroitin/dermatan sulfates and proteoglycans, major constituents of the extracellular matrix in the vessel wall. In VSMC, mesangial cells and endothelial cells, Ang II increases levels and activity of plasminogen activator inhibitor-1, influencing fibrinolysis, extracellular matrix turnover and degradation and regulation of cell migration. Some of these actions have been linked to the AT_4 receptor subtype. However, this remains to be clarified. Ang II also stimulates the activity of matrix metalloproteinases (58) responsible for extracellular matrix degradation. Accordingly, Ang II influences vascular structure by stimulating synthesis of structural components of the extracellular matrix and by increasing production of factors that degrade the extracellular matrix proteins.

Conclusions

Ang II activates multiple signaling pathways. Until recently, the signaling events elicited by Ang II were considered to be rapid, short-lived and divided into separate linear pathways. However, these major intracellular signaling cascades do not function independently and are actively engaged in cross-talk. Downstream signals from the Ang II-bound receptors converge to elicit complex and multiple responses (reviewed in 59,60). Ang II induces a multitude of actions in various tissues, and the signaling events following occupancy and activation of angiotensin receptors are tightly controlled and extremely complex. Furthermore, the fact that Ang II transactivates EGFR, PDGF and IGFR suggests that RTKs are down-

stream targets of AT_1 receptors. Alterations of these highly regulated signaling pathways in cardiovascular cells may be pivotal in structural and functional abnormalities that underlie vascular pathological processes such as hypertension and atherosclerosis. Although there has been significant progress in the last few years in the elucidation of aberrations in Ang II-induced signal transduction in hypertension, we still know very little about the processes that underlie these phenomena, and at what point some pathways become more important than others. With the availability today of molecular and pharmacological tools that allow manipulation of specific signaling molecules, identification of distinct abnormalities in intracellular signaling should be possible. This will further our understanding of the role of Ang II in physiological and pathophysiological processes and will help define novel therapeutic targets in cardiovascular disease.

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