Chapter 5
Angiotensin II Signaling in Vascular Physiology and Pathophysiology

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Abstract Initially recognized as a physiologic regulator of blood pressure and body fluid homeostasis, angiotensin (Ang) II has now been shown in innumerable experiments and clinical studies to contribute to the development and maintenance of cardiovascular disease. Dissection of its signaling mechanisms over the past decades has led to the discovery of several novel concepts, such as tissue-specific metabolism of Ang peptides. Identification and cloning of the various receptors through which Ang II acts on almost all tissues has led to the development of specific pharmacologic inhibitors with proven clinical benefit in patients with cardiovascular disorders. Work on the G-protein-coupled Ang II Type 1 receptor has demonstrated that different receptors interact through oligomerization, compartmentalization, and transactivation, and may explain how Ang II can activate G-protein-independent pathways. Unraveling the downstream effects of Ang II in specific cell types corroborates the importance of the cellular redox state on certain signaling pathways. Finally, the effects of Ang II on cell function and phenotype, such as the expression of inflammatory cytokines and receptors promoting the recruitment of inflammatory cells into vascular tissues, have indicated its role in local inflammation as a general pathogenetic basis of cardiovascular disease. The recognition of Ang II as a contributor to such fundamental pathophysiologic mechanisms, which are believed to be a common pathway for diverse cardiovascular risk factors like hypertension and diabetes, has greatly advanced our knowledge of pathologic signaling in vascular tissues and may help to eventually define novel targets for pharmacologic interventions.

The Renin–Angiotensin System (RAS)

Classic RAS

The canonical renin–angiotensin system (RAS) is a circulating hormonal system that controls systemic Ang II production. The octapeptide Ang II was first

discovered in the 1940s as the actual pressor substance in animal models of renal hypertension, and is considered the primary effector molecule of the RAS, regulating blood pressure by vasoconstriction and fluid homeostasis (Braun-Menendez et al. 1940; Page and Helmer 1940). Generation of Ang II occurs in two enzymatic steps. The aspartyl protease renin, which is produced by specialized juxtaglomerular smooth muscle cells in the kidneys in response to reduced renal perfusion pressure or salt depletion, catalyzes the first and rate-limiting reaction. By splitting a Leu-Val peptide bond in humans or a Leu-Leu bond in other species, it releases the final ten N-terminal amino acids from its only known substrate, angiotensinogen (AGT). AGT is a liver-derived α_2-globulin that belongs to the family of serine-protease inhibitors (serpines), and its concentration is the major determinant of systemic renin activity. The peptide fragment resulting from cleavage of AGT, Ang I, is the inactive precursor of Ang II. Ang II is generated by cleavage of two C-terminal amino acids (His-Leu) from Ang I by the dipeptidyl carboxypeptidase angiotensin-converting enzyme (ACE) that is anchored to the luminal surface of the endothelium as a transmembranous ectoenzyme and is particularly abundant in the pulmonary vasculature (Caldwell et al. 1976).
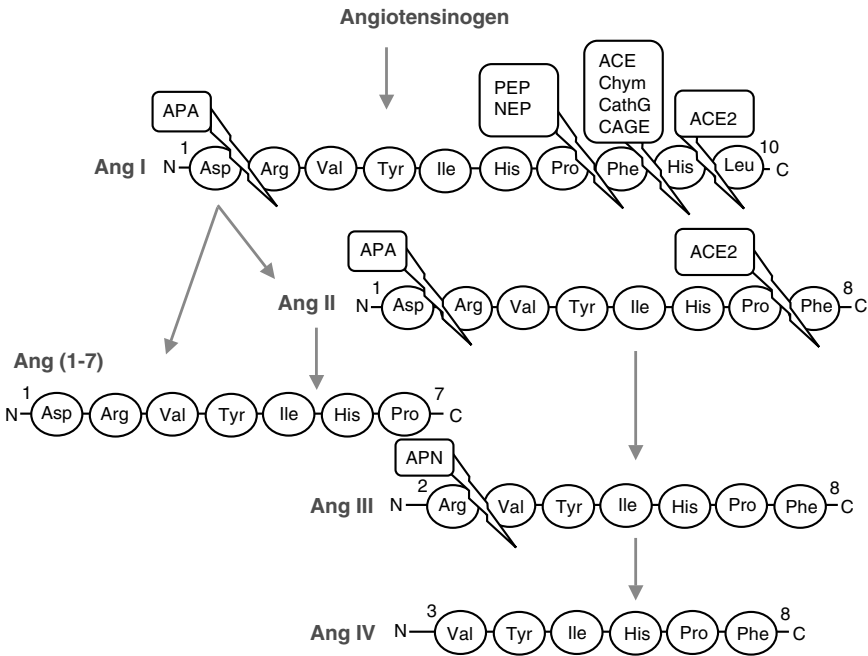
Fig. 5.1 Metabolism of angiotensin peptides. Various peptidases regulate the production and degradation of diverse angiotensin peptides, accounting for tightly regulated agonist and antagonist functions systemically and locally. ACE, angiotensin-converting enzyme; Ang, angiotensin; APA, aminopeptidase A; APN, aminopeptidase N; CAGE, chymostatin-sensitive angiotensin II-generating enzyme; Chym, chymase; CathG, cathepsin G; NEP, neutral endopeptidase; PEP, prolylendopeptidase.

Recent advances in the metabolism of angiotensin peptides have revealed novel functional peptides, which challenge the view of Ang II as the primary effector of the RAS (Figure 5.1). Aminopeptidase A can split off Asp from Ang II, yielding the less potent vasoconstrictor Ang III (Ang 2–8), which can further be transformed to Ang IV (Ang 3–8) by aminopeptidase N. Another angiotensin peptide, Ang 1–7, which is derived either directly from Ang I by tissue-endopeptidases or from Ang II by ACE2, acts as a vasodilator (Ferrario and Iyer 1998). ACE2 is an ACE homologue that acts as a carboxypeptidase by selectively removing the C-terminal Phe from Ang II. Through its contribution to Ang II degradation as well as Ang 1–7 generation, ACE2 has received much attention as an opposing factor of Ang II action. To highlight the heterogeneous functions of the various RAS components, ACE2 has also been recognized as a functional receptor for the SARS coronavirus, mediating its cell intrusion (Kuhn et al. 2004).

Local Tissue RAS

The vascular wall possesses the ability to independently generate Ang II, suggesting local paracrine/autocrine functions of Ang II. Except for renin, all components of the RAS have been found in the vascular wall. The rate-limiting step of local Ang II generation is the conversion of Ang I to Ang II, which can be catalyzed by a number of enzymes in addition to ACE, namely, chymase, cathepsin G, and chymostatin-sensitive Ang II-generating enzyme (CAGE). Several reninlike enzymes that release Ang I from AGT, such as cathepsin D, tonins, tissue plasminogen activator, and other aspartyl proteases, have been proposed as a means to mediate the local RAS independent of kidney-derived renin. While there is no convincing evidence for local synthesis of renin (Hilgers et al. 2001), tissue levels of renin become undetectable within 48 hours after bilateral nephrectomy (Thurston et al. 1979). This suggests accumulation of renin in tissues. Renin uptake into cardiovascular tissues has been reported to result in sustained Ang II effects in local vascular beds (Müller et al. 1998). Furthermore, a functional renin receptor has recently been found on mesangial cells that enhances the catalytic activity of renin upon binding (Nguyen et al. 2002). This receptor specifically binds renin and its precursor prorenin, which may explain why local concentrations of renin (e.g., in interstitial fluid) are oftentimes higher than predicted by simple diffusion from the plasma (Danser et al. 1994).

Angiotensin II Receptors

The cellular effects of Ang II are mediated by distinct high-affinity cell surface receptors. Two subtypes can be discriminated by specific nonpeptide antagonists. Additionally, exposure to the sulfhydryl-reducing dithiothreitol (DTT) inactivates Ang II Type 1 receptors (AT_1Rs), whereas Ang II Type 2 receptors (AT_2Rs) remain

intact (Chiu et al. 1989). The AT_1R was initially cloned from rat aortic smooth muscle and bovine adrenal cells (Murphy et al. 1991; Sasaki et al. 1991), while the AT_2R was cloned from a rat pheochromocytoma cell line and from fetal rat tissue (Kambayashi et al. 1993; Mukoyama et al. 1993).

Additional receptor subtypes (e.g., AT_3R and AT_4R) have been described pharmacologically. Based on binding studies in the brain, the AT_4R was originally defined as the binding site for the hexapeptide Ang IV. Later on, the AT_4R was found to be identical to insulin-regulated aminopeptidase (IRAP), a protein first identified as membrane-bound metalloprotease in insulin-sensitive GLUT4 vesicles of fat and muscle cells (Keller et al. 1995). Ang IV binds and inhibits IRAP and enhances cognitive functions in experimental animals (Chai et al. 2004).

The human AT_1R and AT_2R are single-copy genes, but rodents have two AT_1R gene (AGTR1) loci (AGTR1a on chromosome 17; AGTR1b on chromosome 2) and accordingly express two AT_1R isoforms ($AT_{1A}R$ and $AT_{1B}R$) that differ in 18 amino acids, mainly in the carboxy-terminal region. Overall, they are 94% homologous and pharmacologically indistinguishable (Iwai and Inagami 1992). $AT_{1A}Rs$ are expressed predominantly in vascular smooth muscle, endothelial cells, liver, lung, kidney, brain, ovary, and testis, whereas $AT_{1B}Rs$ occur mainly in the adrenal and anterior pituitary gland (Burson et al. 1994). The human AT_1R is $\sim$ 95% homologous to the rodent isoforms (Curnow et al. 1992).

AT_1Rs

Most vascular Ang II effects known to date are mediated by AT_1Rs, the best-characterized Ang II receptor so far. The AT_1R can be blocked by biphenylimidazoles including losartan (DuP 753), valsartan, and candesartan, and is mainly expressed in vascular smooth muscle cells (VSMCs), but also in heart, lung, liver, adrenal cortex, kidney, and brain. Several reports show that Ang II has effects on endothelial cells, but not always do they seem to be mediated by known Ang II receptors (Vaughan et al. 1995). Aside from rodent endothelial cells, which clearly express $AT_{1A}Rs$, AT_1Rs have also been found on bovine and porcine endothelial cells. In primary cultured human arterial umbilical endothelial cells, AT_1Rs have been detected by binding studies; however, the receptors rapidly disappear *in vitro*. Interestingly, a low-affinity binding site persists even in later cell passages, which is not inhibitable by specific receptor blockers (Ko et al. 1997). On the other hand, AT_1Rs have not been found on human endothelial cells using immunohistochemistry (Allen et al. 2000).

Structure and Genomics

The AT_1R is a seven-transmembrane-domain rhodopsinlike peptidergic G-protein-coupled receptor (GPCR) that upon ligand binding activates heterotrimeric

G-proteins to direct subsequent signaling events. The AT_1R has 359 amino acids (MW ~50 kDa). Structurally, it consists of four extracellular (N-terminus and three connecting loops), four intracellular (three connecting loops and C-terminus), and seven α-helical transmembrane domains. Extracellular glycosylation sites are in the N-terminus (Asn[4]) and the second extracellular loop (Asp[176] and Asn[188]) (Desarnaud et al. 1993). Two disulfide bonds between extracellular loops one and two and between extracellular loop three and the N-terminus stabilize the tertiary structure of the receptor. The N-terminus as well as the first and third extracellular loops contain the epitopes for peptide binding. The binding of nonpeptide antagonists is independent from these epitopes, suggesting distinct modes of interaction for peptide and nonpeptide ligands (Hjorth et al. 1994). Several conserved residues, Asp[74], Tyr[215], and Tyr[292], are important in G-protein binding and activation (Marie et al. 1994). The cytoplasmic tail can be phosphorylated in the basal and Ang II-stimulated state at its numerous Ser and Thr residues, but also at the few Tyr residues at positions 302, 312, 319, and 339 (Kai et al. 1994). Among these the Asp-Pro-Leu-Phe-Tyr (NPLFY[302]) sequence is a variant of the highly conserved tyrosine-containing NPXY motif found in the cytoplasmic tail of many receptor tyrosine kinases (RTKs), in which it is linked to coated pit-mediated receptor internalization. However, in the AT_1R the NPLFY motif is not linked to receptor internalization. Instead, the extra Phe[301] seems to be important for agonist binding (Hunyady et al. 1995). Tyr[319] is also part of a functionally important motif: YIPP. This sequence is similar to motifs in the platelet-derived growth factor receptor (PDGF-R) and epidermal growth factor receptor (EGF-R), which, when phosphorylated, are important in SH2-domain coupling to those receptors. In the AT_1R, Tyr[319] mediates interaction with JAK2 (Ali et al. 1997), a member of the Janus family kinases (JAK), and is also important for EGF-R transactivation.

The human AGTR1 is located on chromosome 3q21–25 and spans about 60 kb, including five exons and four introns. Exon sizes range from 59 to 2014 bp with exon 5 being the largest and the only coding exon, while the first four exons encode the 5′ untranslated region (UTR) (Guo et al. 1994). Several splice variants with different exon composition have been described. The open reading frame of the AGTR1 spans 1080 bp. AGTR1 polymorphisms have been described as a potential link to vascular morbidity. The single nucleotide polymorphism (SNP) A1166C in the 3′ UTR is more common in hypertensive patients than normotensive controls (Bonnardeaux et al. 1994). In addition, patients with coronary artery disease (CAD) who are homozygous for the A1166C mutated allele (CC genotype) have increased AT_1R sensitivity (van Geel et al. 2000). Some other SNPs in the AGTR1 have been implicated in the genetics of hypertension, but much work needs to be done to evaluate the association of other AGTR1 SNPs with cardiovascular disease.

Regulation of Surface Expression

The density of Ang II receptors on VSMCs is a central determinant of the cellular sensitivity to Ang II. Thus, factors that regulate AGTR1 expression have a marked

Table 5.1 Regulation of AT_1R expression in VSMCs

Upregulation	Downregulation
LDL	Angiotensin II
Insulin	Epidermal growth factor
Insulin-like growth factor-1	Fibroblast growth factor
Progesterone	Platelet-derived growth factor
Erythropoietin	α-Thrombin
Interleukin-1α	ATP
Interleukin-6	Interferon-γ
TNF-α	Nitric oxide
C-reactive protein	Reactive oxygen species
Glucocorticoids	HMG-CoA reductase inhibitors
Sodium chloride	PPAR-γ agonists
Hypoxia	Estrogen
Hyperglycemia	Vitamin A
	Thyroid hormone
	All-*trans* retinoic acid
	Forskolin
	Isoproterenol

LDL, low-density lipoprotein; ATP, adenosine triphosphate; TNF, tumor necrosis factor; HMG-CoA, 3-hydroxy-3-methyl-glutaryl-coenzyme A; PPAR, peroxisome proliferator-activated receptor.

effect on cardiovascular function. Among these are cytokines, hormones, growth factors, and vasoactive agents (Table 5.1). In vascular tissues, chronic Ang II exposure itself induces downregulation of the AT_1R in a negative feedback fashion (Nickenig et al. 2000). Both mRNA and protein are significantly downregulated after 2–6 hours of Ang II exposure *in vitro* (Lassegue et al. 1995) and *in vivo* (Gunther et al. 1980). Various pathophysiological conditions affect AT_1R regulation. For example, hypercholesterolemia leads to AT_1R overexpression (Strehlow et al. 2000). This may explain why hyperlipidemia is frequently associated with hypertension. Intriguingly, oxidized low-density lipoprotein (oxLDL), which has been linked to early atherosclerotic events, increases AT_1R expression on endothelial cells (Li et al. 2000), but in contrast to unmodified LDL, it does not affect AT_1R expression on VSMCs (Nickenig et al. 1997). 3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins), which are potent cholesterol-lowering drugs, have been shown to reduce AT_1R expression by reducing the half-life of AT_1R mRNA. This mechanism is dependent on their inhibitory effect on geranylgeranylation and may therefore explain lipid-lowering independent (pleiotropic) effects of statins on the vasculature (Wassmann et al. 2001). Proinflammatory cytokines increase AT_1R expression in vascular tissues (Sasamura et al. 1997), whereas nitric oxide (NO) downregulates AGTR1 transcription (Ichiki et al. 1998).

Stability of AT_1R mRNA depends on specific binding motifs for polysomal proteins in the 3′ UTR immediately upstream of the polyadenylation tract (Nickenig et al. 2001). There are two putative polyadenylation sites in the 3′ UTR

as well as six AUUUA motifs (Furuta et al. 1992). Mutation of the AU-rich element ($3'$ UTR 2159–2175) decreases AT_1R mRNA decay, while AU-repeat mRNA-binding proteins promote AT_1R mRNA destabilization. In addition to the primary base composition, the secondary structure of the mRNA, displaying a stem-loop sequence, seems to be necessary for interaction with degrading mRNA-binding proteins (Berger et al. 2005). Moreover, the $5'$ UTR sequence has been found to be involved in posttranscriptional regulation. Although in all known splice variants the open reading frame is not affected, variants in the $5'$ leader sequence contribute to tissue-specific changes in AGTR1 expression at least in part by altering rates of mRNA translation (Elton and Martin 2003; Zhang et al. 2004).

Initiation of mRNA synthesis is another control point of AGTR1 expression. Aside from binding sites for activator proteins AP-1 and AP-2, and cyclic AMP regulatory element (CRE), there are binding sites for the eukaryotic transcription factor Sp1 in the ATGR1a promoter, including a GC-box-related sequence within the $-58/-34$ region (Takeuchi et al. 1993). Direct binding of the activated peroxisome proliferator-activated receptor (PPAR)-γ to Sp1 causes inhibition of AGTR1a transcription (Sugawara et al. 2001). Another GC-box-related sequence is located at $-98/-79$ of the AGTR1 promoter in rats and humans (Zhao et al. 2000). Both Sp1 binding sites are additively involved in driving basal AGTR1 expression (Kambe et al. 2004). It has also been suggested that increased levels of Sp1 proteins in the hypothalamus contribute to the hypertensive phenotype in spontaneously hypertensive rats (SHRs) via increased AT_1R expression (Kubo et al. 2003). In proximal tubular cells, a *cis*-acting GAGA-box at $-161/-149$ has also been identified as an important regulator for basal and growth factor-induced AGTR1 transcription, suggesting that the AGTR1 may possess alternative initiation sites (Wyse et al. 2000).

Oligomerization

Although a single AT_1R is fully functional, emerging data show that AT_1Rs are capable of forming homo- and heteromers with other GPCRs. Homodimerization of the AT_1R on monocytes correlates with enhanced Ang II signaling and monocyte adhesion to the endothelium (AbdAlla et al. 2004). Coexpression of nonfunctional AT_1R mutants abrogates coupling to G-proteins, whereas recruitment of β-arrestins and subsequent stimulation of the mitogen-activated protein kinase (MAPK) pathway remains intact suggesting, that oligomerization of intact AT_1Rs is particularly pertinent for G-protein signaling (Hansen et al. 2004). Receptors that dimerize with the AT_1R include the bradykinin B_2 receptor, the β_2-adrenergic receptor, the dopamine D_1 receptor, and the AT_2R (AbdAlla et al. 2000, 2001; Barki-Harrington et al. 2003; Zeng et al. 2003). The AT_1R/B_2 heterodimers confer increased Ang II sensitivity, and an increased number of these heterodimers has been clinically correlated with preeclampsia (Quitterer et al. 2004). It has not been conclusively determined if oligomerization is affected by ligand binding, since some data suggest that it may occur even before surface expression (Hansen et al. 2004). Oligomerization may differentially couple the AT_1R to specific downstream signaling proteins,

or change receptor ligand binding characteristics or receptor trafficking patterns, all of which may integrate information from multiple receptors and modulate Ang II sensitivity of cells.

Internalization and Trafficking

A characteristic consequence of AT_1R activation is cellular desensitization to Ang II. Within 10 minutes, the receptor is internalized via clathrin-coated pits into endosomes. The intracellular Ang II/AT_1R complex continues to signal until its inactivation, which occurs either by degradation after fusion with lysosomes or by dephosphorylation with subsequent recycling to the cell surface membrane (Hunyady et al. 2002). Approximately 25% of the internalized receptors are recycled back to the cell membrane. Internalization and cellular processing of the Ang II/AT_1R complex itself may have a specific role in signal transduction by engaging the complex with second messenger systems other than those at the cell membrane. The principal mechanism depends on Ser- and Thr-phosphorylation of the activated AT_1R C-terminus mediated by G-protein-related kinases (GRKs). Phosphorylation in the region from Thr^{332} to Ser^{338} facilitates interaction with β-arrestins (Qian et al. 2001), which impair further G-protein activation and subsequently promote receptor endocytosis (Kule et al. 2004). β-Arrestins recruit non-receptor tyrosine kinases (NRTKs) like c-Src and other adapter proteins to the cell membrane to form complexes that orchestrate the internalization process (Fessart et al. 2005). After receptor phosphorylation by GRKs 5/6, β-arrestins can direct signaling events toward the MAPK pathway independent of G-proteins (Kim et al. 2005b). For instance, mutant AT_1Rs incapable of G-protein coupling have been found to activate ERK via β-arrestin-2 (Wei et al. 2003). Of interest, hypertension per se upregulates GRK5 in VSMCs *in vivo*, suggesting that modulation of GRK5 levels may be an adaptive cellular means to autoregulate Ang II sensitivity (Ishizaka et al. 1997). After endocytosis, the AT_1R remains tightly bound to β-arrestin and travels to an early sorting endosome, from which the complex fuses either to a lysosome or to a perinuclear recycling endosome (Gaborik and Hunyady 2004). Sorting of the receptor–ligand complex between intracellular membrane organelles is an important trafficking mechanism. It has been shown that AT_1Rs preferentially traffic to Rab5-positive endosomes (Seachrist et al. 2002). Rab GTPases regulate intracellular vesicle transport and fusion. Furthermore, Rab5 and β-arrestin binding to the AT_1R C-terminus appear to mediate retention of the complex in early endosomes, thereby preventing recycling and degradation (Dale et al. 2004). This could represent a mechanism to prolong Ang II effects intracellularly. Nuclear accumulation of the AT_1R has also been described with effects on cellular proliferation, and it has been speculated that the AT_1R directly participates in transcriptional regulation (Cook et al. 2006).

Interestingly, direct microinjection of Ang II into VSMCs with concomitant extracellular AT_1R blockade has been demonstrated to generate an intracellular calcium increase. However, concomitant microinjection of an AT_1R blocker abolished this Ang II effect (Haller and Luft 1998). This confirms a functional coupling of

Ang II and AT_1R intracellularly as seen in the internalization process described above, but direct actions of free intracellular Ang II, which either has escaped from the internalized AT_1R–Ang II–ligand complex or has been synthesized *de novo*, have also been hypothesized. The functional relevance of this mechanism *in vivo* remains to be determined.

Microdomains and Lipid Rafts

It has become apparent that the lipid bilayer cell membrane has distinct regional characteristics with different functions particularly related to cell signaling. Regions that are predominantly composed of sphingolipids and cholesterol in the outer lipid layer, called lipid rafts, are regions of concentrated signaling proteins such as G-proteins, suggesting that the lipid composition surrounding a GPCR may influence receptor function. Caveolae are special invaginated lipid rafts characterized by the presence of caveolin (Cav)-1, -2, and -3, which are of particular importance for Ang II signaling. Upon stimulation with Ang II, the AT_1R rapidly (<2 min) translocates laterally to Cav-enriched membrane fractions, and directly interacts with Cav-1, promoting the assembly of a Cav scaffolding domain (Ishizaka et al. 1998). This process requires a functional cytoskeleton and cAbl, an actin-binding NRTK, to direct proper translocation of AT_1R into lipid rafts with subsequent Rac1 and NADPH oxidase activation, which eventually mediates VSMC hypertrophy (Zuo et al. 2005). It is possible that the AT_1R complex is also internalized at these noncoated caveolae.

AT_2R

The AT_2R has 363 amino acids (MW ∼44 kDa) with only ∼30% homology to the AT_1R sequence. The AGTR2 is located on the X chromosome. It is mainly expressed in uterine smooth muscle, brain, ovary, adrenal medulla, heart, and fetal mesenchyme, and is specifically antagonized by tetrahydroimidazopyridines like PD123319 and PD123177 (EXP655) (Wharton et al. 1998). Its wide expression in fetal tissues supports the concept that it has modulatory functions during embryonic development; however, AT_2R surface expression increases under certain pathological conditions. For instance, in heart failure patients the ratio of AT_2R to AT_1R in the heart increases (Tsutsumi et al. 1998). Additionally, it has been reported that AT_2R is reexpressed in vascular inflammation and injury (Akishita et al. 2000). Interestingly, gene targeting of the AT_2R blocks cardiac hypertrophy and fibrosis in mice with Ang II-induced hypertension (Ichihara et al. 2001). The AT_2R is also present on bovine pulmonary endothelial cells where it increases endothelial NO production and counterbalances AT_1R-mediated vasoconstriction in the setting of hypoxia (Olson et al. 2004). In contrast to the AT_1R, the AT_2R does not undergo receptor internalization (Hunyady et al. 2004). The third intracellular loop seems to

carry the unique features of the AT_2R in regards to G-protein interaction (Lehtonen et al. 1999). AGTR2 expression is also regulated by multiple extracellular factors. Interestingly, Ang II infusion upregulates vascular AT_2R expression in mesenteric arteries of rats (Bonnet et al. 2001).

Signaling Pathways of the AT_1R

Contraction

Ang II activates a complex series of signaling events that are temporally and spatially tightly controlled. Some second messenger systems are activated within seconds, while others are delayed and persist for more than an hour (Griendling et al. 1986). Upon Ang II binding to VSMCs, the AT_1R activates G-proteins of the pertussis toxin-insensitive subfamilies: $G\alpha_q$ and $G\alpha_{12/13}$. Within 30 sec of Ang II stimulation, a rapid and transient activation of phosphatidylinositol-specific membrane-bound phospholipase C (PLC)-β1 leads to the production of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). $G\alpha_{q/11}$ and $G\alpha_{12}$ proteins as well as their respective Gβγ subunits couple the AT_1R with PLC-β1 (Figure 5.2). Subsequently ($\geq$30 sec), PLC-γ activation accounts for the majority
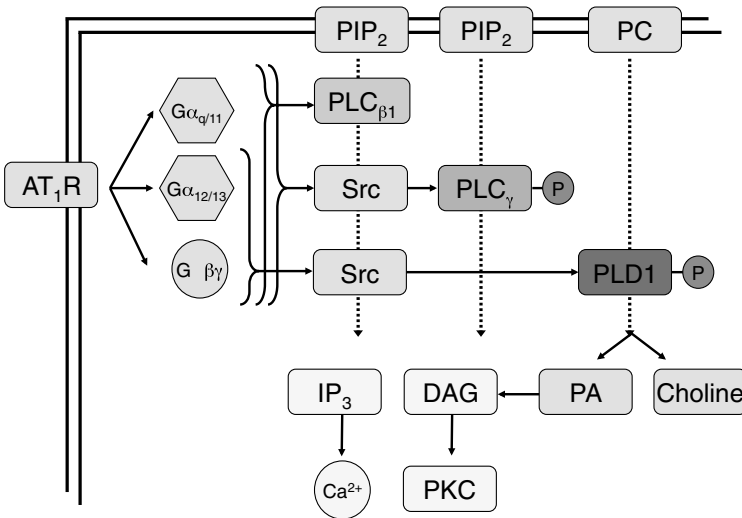
Fig. 5.2 Early G-protein signaling events at the AT_1R. Phospholipases C and D are sequentially activated by heterotrimeric G-protein subunits to produce important second messengers such as IP_3 and DAG. See text for details. AT_1R, angiotensin II type 1 receptor; DAG, diacylglycerol; IP_3, inositol 1,4,5-trisphosphate; PA, phosphatidic acid; PC, phosphatidylcholine; PIP_2, phosphatidylinositol-4,5-bisphosphate; PKC, protein kinase C; PLC, phospholipase C; PLD, phospholipase D.

of IP$_3$ production. PLC-γ is activated in a tyrosine kinase-dependent manner and returns to baseline activity in 10 min (Ushio-Fukai et al. 1998b). Some evidence exists to suggest the NRTK Src as the responsible tyrosine kinase in this step (Haendeler et al. 2003). IP$_3$ rapidly diffuses to the endoplasmic reticulum (ER) where it binds to IP$_3$ receptors to release calcium (Ca^{2+}) into the cytosol. In addition to IP$_3$-triggered release of Ca^{2+} from intracellular Ca^{2+} stores, sustained Ang II-induced vasoconstriction depends on transmembrane Ca^{2+} influx (Ruan and Arendshorst 1996). The production of the membrane-bound DAG by Ang II is biphasic. The initial peak at 15 sec results from hydrolysis of phosphoinositides by PLC, whereas the second phase, which has its maximum at 5 min, results from phospholipase D (PLD) activation. Gα_{12} and G$\beta\gamma$ activate PLD through c-Src and RhoA-dependent mechanisms. PLD remains active for at least 1 hour, splitting phosphatidylcholine into choline and phosphatidic acid (PA) (Ushio-Fukai et al. 1999a), which is subsequently converted to DAG. For this prolonged phase of DAG production, internalization of the ligand-bound AT$_1$R complex is required, implying that PLD activation occurs spatially separated from the plasma membrane. DAG, in conjunction with phosphatidylserine and Ca^{2+}, subsequently activates protein kinase C (PKC). The early increase in intracellular Ca^{2+} stimulates the Ca^{2+}/calmodulin-dependent myosin light chain kinase to phosphorylate Ser[19] of the myosin regulatory light chain (MLC) with subsequent VSMC contraction through MLC interaction with actin. This process is importantly modulated by the Ca^{2+} sensitivity of the myofilaments, which is largely determined by Rho-associated kinase (ROCK)-mediated inhibition of MLC phosphatase (Uehata et al. 1997).

In addition, Ang II rapidly phosphorylates and activates PLA$_2$ to produce arachidonic acid (Rao et al. 1994). Cytochrome P450 metabolites of arachidonic acid, such as 20-hydroxyeicosatetraenoic acids (20-HETEs), have been implicated in vasoconstriction in various vascular beds (Roman 2002).

Controlled relaxation following contraction is vital to the dynamic regulation of blood vessel diameter and hence flow. Termination of Ang II signaling is achieved by regulator of G-protein signaling-2 (RGS2). Ang II upregulates RGS2 mRNA in a PKC-dependent manner thereby providing functional negative feedback (Grant et al. 2000). RGS2-deficient mice have dramatically increased blood pressure due to a prolonged response to Ang II in resistance vessels, as evidenced by the fact that their hypertension is rapidly reversed by Ang II blockade (Heximer et al. 2003).

Cell Growth

Tissue growth is characterized by cellular hypertrophy with increased protein translation, and cellular hyperplasia (proliferation) with increased DNA synthesis, cell cycle progression, and presumably inhibition of cell death pathways. This latter response eventually depends on regulation of gene expression by transcription factors. Although extracellular signals regulating cell growth have classically been
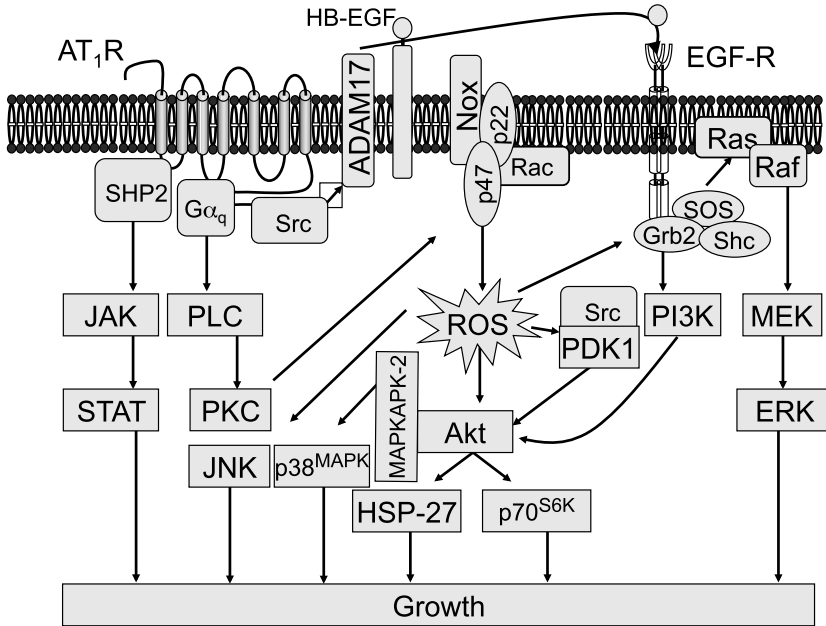
Fig. 5.3 Angiotensin II stimulates vascular smooth muscle cell growth.Growth-promoting effects of Ang II are largely mediated by EGF-R transactivation and stimulation of tyrosine kinase signaling cascades. See text for details. ADAM, a disintegrin and metalloproteinase; AT_1R, angiotensin II type 1 receptor; EGF-R, epidermal growth factor receptor; HB-EGF, heparin-binding epidermal growth factor; HSP, heat shock protein; JAK, Janus kinase; JNK, c-Jun NH_2-terminal kinase; PKC, protein kinase C; PI3K, phosphatidylinositol 3-kinase; PDK1, 3-phosphoinositide-dependent kinase-1; ROS, reactive oxygen species.

considered to be mediated by growth factors and cytokines with activation of RTKs and NRTKs, Ang II has been found to be a potent growth stimulus for VSMCs (Figure 5.3). This is reflected by an increase in cell volume and protein content (Berk et al. 1989; Geisterfer et al. 1988), whereas a proliferative effect of Ang II has been found to be dependent on the cellular milieu (e.g., autocrine production of transforming growth factor-β1 can suppress the hyperplastic response) (Gibbons et al. 1992). Within 1 min of Ang II exposure, numerous cytosolic proteins have been found to be tyrosine phosphorylated (Molloy et al. 1993). Furthermore, the growth-promoting effects of Ang II were found to be abrogated by tyrosine kinase inhibitors (Leduc et al. 1995). It is now well accepted that Ang II rapidly activates the MAPK family, including c-Jun NH_2-terminal kinase (JNK), also termed stress-activated protein kinase, extracellular signal-regulated kinases (ERK, $p42/44^{MAPK}$)1/2, and $p38^{MAPK}$, which are considered upstream mediators of transcription factors involved in VSMC growth.

ERK1/2 activation by Ang II, which can be blocked by PLC inhibition, intracellular Ca^{2+} chelation, and tyrosine kinase inhibition, eventually proceeds through a Ras-dependent pathway (Eguchi et al. 1996). One intermediate tyrosine kinase

between AT_1R and Ras is Src (Schieffer et al. 1996). Growth factor receptors, which are typically RTKs such as EGF-R, characteristically propagate their signal to the small GTPase Ras via the GTP-exchange factor son of sevenless (SOS), which forms multimeric signaling complexes with the receptor and adapter proteins such as Grb2 and Shc. Importantly, it has been demonstrated that the atypical PKC-ζ mediates Ang II activation of Ras, which seems to be a unique aspect of the Ang II-induced response as compared to other growth factors (Liao et al. 1997). Ang II induces EGF-R activation by an intracellular Ca^{2+} – and reactive oxygen species (ROS)-dependent mechanism, which has been termed EGF-R transactivation (Eguchi et al. 1998; Ushio-Fukai et al. 2001), and the EGF-R then acts as a scaffold, mediating a number of downstream signaling molecules including phosphatidylinositol 3-kinase (PI3-K) and Akt. Src recruits another NRTK of the focal adhesion kinase (FAK) family, called proline-rich tyrosine kinase (Pyk) 2, and after assembling a signaling complex, activates subtype ADAM17 of the *a d*isintegrin *a*nd *m*etalloprotease (ADAM) family to release cell surface-bound EGF, which subsequently activates its receptor (Eguchi et al. 1999; Ohtsu et al. 2006; Prenzel et al. 1999) and Ras. An important consequence of Ang II-induced ERK1/2 activation in conjunction with PI3-K activity is an increase of protein translation by PHAS-1-eukaryotic initiation factor-4E (Rocic et al. 2003).

Interestingly, EGF-R transactivation is only required for ERK1/2 and p38^{MAPK} activation, but not for JNK activation (Eguchi et al. 2001). A parallel and independent mechanism of ERK1/2 activation by Ang II occurs via β-arrestin-2 as mentioned above (Wei et al. 2003). Apoptosis signaling-related kinase (ASK) 1 is required for JNK and p38^{MAPK} activation (Tobiume et al. 2001), while JNK activation also depends on Rho/ROCK activation and subsequent rac stimulation (Ohtsu et al. 2005). Activation of p38^{MAPK} by Ang II leads to the stimulation of MAPKAPK-2, heat shock protein-27 (HSP-27), and Akt (Taniyama et al. 2004), the latter of which is crucial for VSMC hypertrophy.

JAKs, which are classically activated by cytokine receptors, are upstream controllers of the transcription factors signal transducers and activators of transcription (STATs), which in turn regulate transcription of early growth response genes such as *c-fos, c-myc*, and *c-jun* (Horvath and Darnell 1997). JAK2 binding to Tyr^{319} of the AT_1R is facilitated by SH2 domain-containing tyrosine phosphatases (SHPs) (Marrero et al. 1998).

Several aspects of the growth-promoting pathways have a special characteristic of being ROS-dependent, which means that enzyme activity is susceptible to oxidation. In fact, many signaling proteins, such as transcription factors and protein tyrosine phosphatases (PTPs), rely on reduced Cys residues for activity. Ang II via its AT_1R generates ROS via a membrane-bound multisubunit protein complex, called NADPH oxidase (Nox) (Griendling et al. 1994). Arachidonic acid metabolites play an important role in Nox activation (Zafari et al. 1999). ROS, in turn, modify critical signaling cascades such as p38^{MAPK} (Ushio-Fukai et al. 1998a). Several different isoforms of Nox have now been identified that differ in subunit composition, subcellular localization, and probably type of ROS produced (Bedard and Krause 2007; Hilenski et al. 2004). Different ROS may have

distinct vascular effects. For instance, vascular hypertrophy in Ang II-mediated hypertension is mediated by VSMC-derived H_2O_2, while a main effect of superoxide ($O_2^{\cdot-}$) is to inactivate antiatherogenic NO (Rajagopalan et al. 1996; Zhang et al. 2005c).

Tissue growth also occurs through expansion of noncellular components. Chronic Ang II stimulates collagen production (Kato et al. 1991). This observation is frequently referred to as transformation of VSMCs into a synthetic phenotype, which then produce proteoglycans, glycosaminoglycans, collagen type 1, and fibronectin, all of which are constituents of the extracellular matrix. The synthetic phenotype of VSMCs is predominant in hypertensive animals, whereas their normotensive littermates contain more VSMCs of the contractile phenotype (Fukuda et al. 1999). At the same time, VSMCs modulate extracellular matrix degradation by activation of matrix metalloproteinases (MMPs). Extracellular matrix degradation is a prerequisite for cell migration on the one hand, but on the other it has been implicated in plaque instability.

Cell Migration

Ang II-induced VSMC migration is strongly implicated in atherosclerosis and restenosis after vascular injury, because abnormal accumulation of VSMCs in the vessel intima entails movement out of the media (Prescott et al. 1991). It was suggested that VSMCs migrate from the media to the intima as a result of a PDGF gradient (Okuda et al. 1995). *In vitro* studies further confirmed that Ang II is also capable of stimulating VSMCs to migrate, although less potently than PDGF (Bell and Madri 1990), and that NO antagonizes this effect (Dubey et al. 1995). A role for Ang II in the neointima formation of restenosis was suggested based on studies showing that tissue ACE activity is elevated after vessel injury and that ACE inhibition can prevent restenosis (Rakugi et al. 1994), although the relevance of this observation to human disease has been questioned.

Ang II induces VSMC migration by recruiting growth pathways such as the EGF-R–MAPK pathway (Saito et al. 2002; Xi et al. 1999). Additionally, cell migration requires dynamic reorganization of the cytoskeleton and focal adhesion complexes, a process that involves Src activity in VSMCs (Ishida et al. 1999). Ang II stimulates the phosphorylation of FAK, Pyk2, and paxillin (Eguchi et al. 1999; Leduc and Meloche 1995; Okuda et al. 1995), which regulate focal adhesion dynamics. Together with Src, Pyk2 also leads to tyrosine phosphorylation of PDK1, which is critical for focal adhesion assembly (Taniyama et al. 2003). JNK has emerged as key mediator of cell migration through its interaction with these cytoskeleton components and focal adhesion-associated proteins (Huang et al. 2003). The JNK pathway can be activated by the small G-protein Rho and its effector ROCK through PKC-δ and Pyk2 (Ohtsu et al. 2005). Ang II itself is a weak migratory factor, but importantly, it enhances the ability of VSMCs to migrate toward PDGF-BB, possibly by inducing focal adhesions (Dubey et al. 1995).

Apoptosis

A specific suicide capability, apoptosis, allows tissues to regulate their own cell number, which is an essential process in atherosclerosis and restenosis (Isner et al. 1995). Indeed, many growth-promoting factors have been found to also suppress cell death, which may contribute to the deleterious accumulation of VSMCs in the intimal space. Ang II, via its AT_1R, directly inhibits cGMP-induced apoptosis in VSMC (Pollman et al. 1996) by upregulating an enzyme that degrades cGMP, phosphodiesterase 5A (PDE5A) (Kim et al. 2005a). Another mechanism by which Ang II exerts antiapoptotic effects is via stimulation of Noxes to generate H_2O_2, which in turn stimulates antiapoptotic MAPK and Akt activity (Ushio-Fukai et al. 1999b). Conversely, in addition to its antiapoptotic function, Ang II can cause a delayed apoptosis in the epithelioid-shaped subtype of VSMC, which has been suggested to contribute to locally heterogeneous plaque weakening and rupture (Bascands et al. 2001). Apoptosis alone does not cause inflammation in normal arteries, but in atherosclerotic lesions it does, leading to plaque vulnerability (Clarke et al. 2006). Thus, apoptosis is considered both beneficial and detrimental.

Proapoptotic actions of Ang II seem to be mediated substantially by AT_2R activation. It has been demonstrated *in vivo* that AT_2Rs mediate negative vascular remodeling by inducing VSMC apoptosis (Yamada et al. 1998). While ACE inhibitors (ACE-I) block this effect, AT_1R blockers (ARBs) do not (Tea et al. 2000). Interestingly, overexpression of the AT_2R alone stimulates apoptosis in the absence of Ang II (Miura and Karnik 2000). It has been found that PTPs like SHP-1 and MAPK phosphatase (MKP)-1 are linked to the AT_2R via Gi protein coupling to the unique third intracellular loop of the receptor (Lehtonen et al. 1999); however, the signaling pathways linked to the AT_2R have not been well characterized yet.

Inflammation

Numerous studies have shown that VSMCs are capable of producing cytokines in the vessel wall. Cytokines are considered the main modulators of inflammatory events during atherogenesis. Ang II, like inflammatory cytokines, activates proinflammatory transcription factors (Brasier et al. 2000). The resulting inflammatory gene products can be grossly subdivided into adhesion molecules, cytokines, and acute phase reactants. The prototypical Ang II-inducible transcription factors, such as AP-1, STATs, and nuclear factor-κB (NF-κB), are also known cytokine-inducible transcription factors. Ang II dose-dependently stimulates interleukin-6 (IL-6) production in cultured VSMC, which is accompanied by activation of NF-κB. Antioxidants reverse this effect, indicating a role for ROS (Kranzhofer et al. 1999). Ang II increases IL-6 expression as early as 15 min, peaking at 1 h and falling thereafter, whereas NF-κB activation is biphasic with peaks at 15 min and 24 h (Han et al. 1999). Cell cycle regulatory genes, like *AP-1* and *c-myc*, are stimulated via the AP-1 transcription factor (Naftilan et al. 1989; Taubman et al. 1989). Ang II also stimulates transcription of intercellular and vascular adhesion molecules (ICAM,

VCAM) in a NF-κB-dependent manner (Tummala et al. 1999). The link between AT$_1$R and NF-κB activation involves direct phosphorylation of the p65 subunit of NF-κB by ribosomal S6 kinase (RSK) and by IKK, and both pathways appear to be redox-dependent (Zhang et al. 2005a,b).

In addition to NF-κB, Ang II-induced vascular inflammation is critically regulated by the ETS family of transcription factors such as Ets-1. Systemic administration of Ang II to Ets$^{-/-}$ mice has been associated with a marked reduction in medial hypertrophy and recruitment of inflammatory cells into the vascular wall compared to that in wild-type mice, independent of the blood pressure effect (Zhan et al. 2005).

It is therefore likely that the proinflammatory milieu in atherosclerotic lesions is at least in part a consequence of Ang II-induced inflammatory gene expression. Ang II-stimulated production of VCAM-1 and chemokine monocyte chemotactic protein-1 (MCP-1) enhances leukocyte accumulation in the vessel wall. MCP-1 is thought to function locally by establishing a chemical gradient to attract monocytes and T lymphocytes expressing the CC chemokine receptor-2 (CCR2). It has been found that C-reactive protein (CRP), a known systemic acute phase reactant, is synthesized by VSMCs in response to inflammatory cytokines and Ang II (Peng et al. 2006). In turn, CRP upregulates AT$_1$R expression, creating a vicious cycle of proinflammatory signals. Furthermore, CRP has been established as useful biomarker for cardiovascular risk stratification.

Physiological Vascular Functions

The main direct physiological function of Ang II in the vasculature is acute regulation of vascular tone, thereby controlling blood flow to various vascular beds. The classic RAS functions as an adaptive mechanism to maintain circulatory homeostasis, regulating blood pressure acutely by vasoconstriction and chronically by releasing aldosterone and expansion of intravascular volume. Effects of Ang II outside of the vasculature include modulation of sympathetic tone and dipsogenic stimulation to maintain vegetative autoregulatory functions of the vasculature. In the adult vessel, VSMCs are present in the quiescent contractile phenotype until an insult triggers a change in phenotype. Ang II seems to mediate this transformation, which may be adaptive and beneficial in the short term, but in the long term sustains unfavorable and unstable vessel architecture prone to increase the likelihood for adverse clinical events.

Pathophysiologic Functions

Hypertension

Patients with essential hypertension can be divided into subgroups with low and high plasma renin activity, with distinct pathophysiological features. Low-renin or
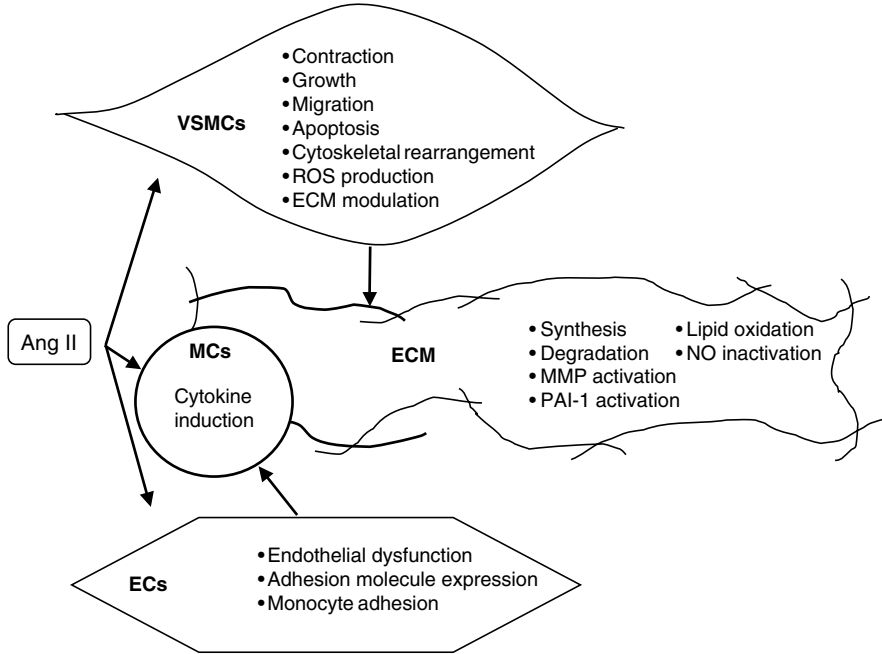
Fig. 5.4 Angiotensin II mediates multiple physiological and pathophysiological responses in the vessel wall.The vessel wall undergoes numerous structural and functional changes mediated by the AT$_1$R, all of which are considered proatherogenic once physiological thresholds are crossed. See text for details. Ang II, angiotensin II; EC, endothelial cell; MC, monocyte/macrophage; MMP, matrix metalloprotease; NO, nitric oxide; PAI-1, plasminogen activator inhibitor-1. ECM, extracellular matrix; ROS, reactive oxygen species.

renin-independent hypertension is characterized by salt-sensitivity, good response to diuretics, and familial aggregation (Fisher et al. 2002). Blockage of the RAS is most effective in hypertensives with high plasma renin activity, whereas it has only limited effectiveness in low-renin states. Patients with hypertension have a multitude of structural and functional disturbances affecting all compartments and cell types of the vascular wall. In particular, increased media thickness has been consistently found in vessels of hypertensive animals and can be attenuated by Ang II inhibition (Rizzoni et al. 1998). This increased media thickness is due in part to hypertrophy of the VSMCs themselves, but also to an increased production of extracellular matrix (Lee 1987). As described above, Ang II has the potential to increase both of these components by its effects on VSMC growth and synthetic capacity (Figure 5.4). In addition to these trophic effects on the vessel wall implicated in chronic hypertension, prolonged contraction of resistance arteries (as illustrated above for the RGS$_2^-$ mouse) and endothelial dysfunction are additional mechanisms of hypertension linked to RAS activity. Ang II-induced O$_2^-$ from VSMC Nox enzymes reacts with endothelial-derived NO leading to impaired endothelial-dependent vasorelaxation (Rajagopalan et al. 1996). Aside from the vessel wall, Ang II also activates Noxes in various other tissues. Recently, it has been shown that activation of

circulating T cells is essential for Ang II-induced hypertension, implying that circulating cells may provide a link to this widespread pattern of Nox activation and ROS production in various tissues and organs (Guzik et al. 2006).

Metabolic Syndrome/Insulin Resistance

Based on the clinical observation that essential hypertension and insulin resistance often coexist, it has been suggested for some time that both entities share common pathogenetic links. The Atherosclerosis Risk in Communities (ARIC) study showed that hypertensive patients are much more likely to develop type 2 diabetes (DM2) than their normotensive peers (Gress et al. 2000). Intriguingly, inhibition of the RAS appears to reduce new onset of DM2 (Yusuf et al. 2000), suggesting a role for Ang II in insulin resistance. Furthermore, diabetic patients benefit overproportionally from RAS inhibition as these agents reduce diabetic nephropathy as well as microvascular and macrovascular complications. Aside from direct functions of Ang II on the endocrine pancreas like control of local blood flow, hormone release, and prostaglandin synthesis, there is substantial evidence that Ang II cross-talks to insulin signaling in VSMCs. Interplay of these two important hormones at the level of VSMCs is an attractive hypothesis for accelerated atherosclerosis found in patients with DM2 or insulin resistance. Observations made *in vitro* suggest that Ang II stimulation of VSMCs leads to serine phosphorylation of the insulin receptor substrate-1, which has been implicated in its premature degradation (Taniyama et al. 2005). Of note, Ang II modulates insulin signaling at multiple levels by serine phosphorylating not only insulin receptor substrate-1, but also the insulin receptor and PI3-K (Folli et al. 1997). In addition, Ang II downregulates the vascular expression of PPARγs (Tham et al. 2002), which are nuclear hormonal receptors and transcription factors that promote beneficial effects on lipid metabolism, insulin sensitivity, and atherosclerosis development. Moreover, they exhibit anti-inflammatory properties by negatively modulating inflammatory gene expression. It is noteworthy that certain ARBs have been shown to directly and AT_1R-independently activate PPARγs in adipocytes, which has been suggested as a mechanism for the insulin-sensitizing/antidiabetic effect of certain ARBs (Schupp et al. 2004).

Atherosclerosis

The development of atherosclerotic lesions involves migration and proliferation of VSMCs. Essentially all of the above-mentioned chronic effects of Ang II on the vasculature are therefore considered atherogenic. While the initiating event for atherogenesis remains elusive, it is clear that a subsequent change in VSMC phenotype maintains disease progression. As opposed to the concentric medial thickening observed in hypertension, atherosclerosis results from the buildup of focal or diffuse

lipid-laden and fibroproliferative plaques in the vessel intima with subsequent narrowing of the vessel lumen. Prior to formation of a plaque, endothelial dysfunction and upregulation of adhesion molecules occurs in atherosclerosis-prone vascular segments (Nakashima et al. 1998). Ang II induces expression of adhesion molecules promoting monocyte invasion into the vasculature. In addition, Ang II not only induces expression of the oxLDL receptor (LOX-1) on macrophages with subsequent macrophage activation, but it also provides the basis for LDL oxidation by stimulating the production of ROS by VSMCs (Griendling et al. 2000). Not surprisingly, Ang II infusion into $apoE^{-/-}$ mice significantly enhances atherosclerotic lesion progression independent of its effect on blood pressure (Weiss et al. 2001). Conversely, Ang II inhibition is known to reduce atherosclerotic lesion formation (Daugherty et al. 2000).

Furthermore, Ang II has been found to contribute to VSMC senescence, which has been implicated in the pathogenesis of atherosclerosis (Kunieda et al. 2006). Cell senescence may promote plaque instability, since loss of VSMCs leads to transformation into a rupture-prone plaque with a thin fibrous cap over the lipid-rich core (Geng and Libby 2002).

Conclusion

Increased RAS activity has emerged as a key mechanism in the pathogenesis of highly prevalent vascular diseases such as hypertension and atherosclerosis. It is therefore vital to understand mechanisms of local Ang II production, Ang II responsiveness, as well as its signaling pathways in normal and disturbed vascular cell function, particularly in VSMCs, the most abundant cell type in the vessel wall. The cellular events initiated by Ang II binding to its AT_1R are complex, with distinct temporal and spatial characteristics, and are far from completely understood. Moreover, cross-talk between the RAS and other metabolic systems such as lipoproteins and insulin may explain common pathogenetic links for cardiovascular diseases. Thus, advancing our knowledge of Ang II signaling in cardiovascular disease bears enormous potential in identifying novel targets for pharmacologic intervention.

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