Intracardiac Intracellular Angiotensin System in Diabetes

Rajesh Kumar, Qian Chen Yong, Candice M. Thomas, Kenneth M. Baker

Division of Molecular Cardiology, Texas A&M Health Science Center, College of Medicine; Scott & White; Central Texas Veterans Health Care System, Temple, TX

Correspondence:

Kenneth M. Baker, M.D., F.A.H.A.
1901 South First Street, Building 205
Temple, Texas 76504
Phone: 254-743-2761
Fax.: 254-743-0165
e-mail: kbaker@medicine.tamhsc.edu
Abstract

The renin-angiotensin system (RAS) has mainly been categorized as a circulating and a local tissue RAS. A new component of the local system, known as the intracellular RAS, has recently been described. The intracellular RAS is defined as synthesis and action of Ang II intracellularly. This RAS appears to differ from the circulating and the local RAS, in terms of components and the mechanism of action. These differences may alter treatment strategies that target the RAS in several pathological conditions. Recent work from our laboratory has demonstrated significant upregulation of the cardiac, intracellular RAS in diabetes, which is associated with cardiac dysfunction. Here, we have reviewed evidence supporting an intracellular RAS in different cell types, Ang II actions in cardiac cells, and mechanism of action, focusing on the intracellular cardiac RAS in diabetes. We have discussed the significance of an intracellular RAS in cardiac pathophysiology and potential therapeutic implications.
Introduction

The renin-angiotensin system (RAS) is an extensively studied hormonal system present in most vertebrates. Starting with the discovery of renin more than a century ago, the RAS has evolved into an extensive system of bioactive peptides that result from the action of several enzymes on a single precursor, angiotensinogen (AGT) (26). These peptides have specific receptors that produce unique cellular responses in multiple tissues. Angiotensin (Ang) II was the first and extensively investigated peptide of the RAS. Initially shown to regulate systemic blood pressure, via modulating salt and water homeostasis and vascular tone, Ang II also produces tissue specific effects that include hypertrophy and fibrosis in the heart (2). The tissue effects of Ang II are generally independent of the systemic effects on blood pressure, which are separated largely due to independent control of Ang II production in the circulation and tissues (61). Thus, Ang II is an endocrine, as well as an autocrine/paracrine hormone, based on the site of synthesis and action (47). Ang II binds primarily to two specific G-protein coupled receptors, Ang type I (AT₁) and Ang type II (AT₂), present on the plasma membrane of most cells. A new aspect of Ang II actions, as an intracrine hormone, has become evident recently. In the intracrine mode, Ang II actions begin from an intracellular location, instead of the interstitial space, similar to a typical peptide-receptor interaction (63). The intracrine aspect of Ang II adds a unique dimension to the RAS, which is significantly different from endocrine and autocrine/paracrine systems, in terms of the site and mode of Ang II synthesis and possible new intracellular receptors and mechanisms of action (78). These differences might potentially change our approach to inhibit the RAS in pathological conditions (46). Diabetes is a disease with predominant
intracrine or intracellular RAS activation and in which classical RAS inhibitors have not been proven as effective as anticipated (86, 93). In this article, we will review studies that describe activation of the intracellular RAS by hyperglycemia, in different cell types, with particular emphasis on the actions of this system in the diabetic heart.

**The Classical RAS**

Several decades ago, the RAS was characterized as a circulating system in which Ang II was generated in the circulation from AGT as a result of sequential cleavage by renin and angiotensin converting enzyme (ACE) (44). The source of AGT in the circulation was mainly the liver, renin was secreted from kidneys, and ACE was primarily enriched on pulmonary endothelial cells. Circulating Ang II has an indisputable role in the pathophysiology of hypertension through multiple mechanisms, which include vasoconstriction, salt and water reabsorption, and aldosterone secretion. In the last two decades, attention has shifted from circulating to local generation of Ang II in tissues, particularly in the brain, heart, vasculature, adrenal glands, pancreas, and adipose tissue. Due to access of circulating Ang II to tissues, it has been difficult to delineate the relative contribution of locally generated versus circulating Ang II to overall tissue concentrations and biological effects. However, utilizing tissue specific genetic models of RAS components and other experimental designs, it is evident that the local RAS has a much greater role in tissue pathology than the circulating RAS (46). Most major organ systems have been reported to express all components of the RAS, which are regulated independent of the circulating system.

**The Cardiac RAS**
All major components of the classical RAS i.e., renin, AGT, ACE, AT₁, and AT₂, are expressed in the heart; although there is a lack of consensus on the source of renin (27, 43). Irrespective of the origin of these components, the majority of Ang II in the heart is produced in situ (87). Cardiac levels of renin and Ang II correlate well with plasma levels before and after nephrectomy in rats; promoting the hypothesis that circulating renin contributes significantly to Ang II production in the heart (9, 17). However, local Ang II levels are increased in pathological conditions such as myocardial infarction (MI) and in the failing heart (10, 71). The difference in circulating and cardiac Ang II levels is particularly prominent in diabetes. Diabetes causes a decrease in circulating renin, while prorenin levels are increased several fold, resulting in a reduced or normal Ang II concentration (38, 74). On the contrary, the tissue RAS activity is significantly increased in diabetes, including in the heart (81). These observations support a local source of RAS components, including renin, in diabetes-induced Ang II production. We have reported increased renin protein and mRNA levels in isolated cardiac myocytes and fibroblasts in response to high glucose exposure (76, 80). In addition, alternative pathways of Ang II synthesis that involve cathepsins and chymase have been described and reviewed elsewhere (45).

As is evident from the foregoing discussion, renin and AGT are secretory proteins and ACE and Ang receptors are localized on the plasma membrane. These characteristics of RAS components dictate that Ang II synthesis occurs outside of cells and ligand-receptor interaction occurs at the level of the plasma membrane, as is typical of a hormonal system. The above is a well-documented and accepted characteristic of the RAS, that defines Ang II as an endocrine and autocrine/paracrine hormone. However,
over the course of several years, investigators have made several observations that suggest an additional mechanism of Ang II functionality, termed an intracrine mechanism (60).

**The Intracellular RAS**

The intracellular RAS and intracrine Ang II might appear to have the same meaning; however, there is a subtle difference between the two. Intracrine Ang II refers to actions of Ang II that originate from an intracellular location, irrespective of the source of Ang II. On other hand, the intracellular RAS denotes the presence of a completely functional RAS inside cells, that includes intracellular Ang II synthesis and action (48).

**Nuclear Ang II and Ang II receptors**

The first suggestion of intracrine functionality of Ang II stemmed from the studies of Robertson and Khairallah, in 1971, who demonstrated nuclear transport of radiolabeled Ang II injected in the left ventricle of adult rats (67). It is now well recognized that binding of extracellular Ang II to plasma membrane AT$_1$ receptor results in internalization of the Ang II-AT$_1$ complex (40, 85). While a significant amount of internalized Ang II is degraded, a regulated portion is targeted to intracellular locations (such as the nucleus), where it could function as an intracrine mediator (31, 51, 72, 88). Specific binding of Ang II to nucleoprotein particles in the chromatin and increased RNA synthesis in isolated rat liver nuclei following exposure to Ang II were also reported (59, 61). We first characterized AT$_1$-like Ang II binding sites on rat liver nuclei (6), which were subsequently shown to induce RNA transcription by others (30). Using electron microscopic and immunofluorescence immunocytochemistry techniques and anti-AT$_1$
antibody, angiotensin II receptors were detected in the sarcolemma, T-tubules, and nuclei of rat cardiomyocytes (37). More recently, both AT\textsubscript{1} and AT\textsubscript{2} receptors were detected on cardiomyocyte nuclei by Western analysis and confocal microscopy. Ang-II application to cardiomyocyte nuclei enhanced NF-κB mRNA expression with approximately a 2-fold greater affinity, in comparison to when applied to intact cardiomyocytes (83). AT\textsubscript{1}-like nuclear Ang II receptors have also been reported in the renal cortex and medulla of rats (55). Isolated nuclei showed increased reactive oxygen species levels following exposure to Ang II, which were abolished by the AT\textsubscript{1} antagonist losartan or the NADPH oxidase (NOX) inhibitor DPI (56). The renal intracellular RAS has been reviewed in more detail elsewhere in this issue. These studies provided a framework for the concept of intracellular actions of Ang II.

**Cellular effects of Ang II**

Some of the first evidence of intracellular effects of Ang II came from studies on cell communication in cardiomyocytes, isolated from adult rats. Intracellular dialysis of Ang I and Ang II decreased junctional conductance in cell pairs, which were suppressed by co-dialysis of an ACE inhibitor and an Ang receptor blocker (ARB), respectively (22). Similar observations were made when the cells were dialyzed with renin or renin plus AGT, suggesting intracellular generation of Ang II by cardiomyocytes (20). It was further demonstrated that endogenous Ang II was involved in the regulation of cell communication in cardiomyocytes, at an advanced stage of heart failure in hamsters, when the cardiac RAS was activated (19). Intracellular Ang II also decreased the cell volume of myocytes isolated from cardiomyopathic hamsters, an effect that was opposite to that of extracellular Ang II (21). In a similar study using vascular smooth
muscle cells (VSMCs), microinjection of Ang II produced an increase in cytosolic and nuclear Ca\(^{2+}\) ions, due to an influx of extracellular Ca\(^{2+}\) ions. This effect of intracellular Ang II could be prevented by microinjection of an ARB, but not by extracellular application (39). In de-endothelized rat aorta rings, intracellular administration of Ang II, through liposomes, resulted in a dose-dependent contraction that was sensitive to an intracellular, but not extracellular ARB (8). Intracellular injection of Ang II increased cytosolic Ca\(^{2+}\) concentration in myometrial cells in a dose-dependent manner, likely through AT\(_1\)-like receptors on lysosomes (25).

More recent studies utilized a recombinant approach to produce Ang II intracellularly. Overexpression of a non-secretory truncated AGT, which resulted in Ang II generation in the cytoplasm, induced cell growth and PDGF expression in rat hepatoma cells (11, 15). We used a recombinant adenoviral expression vector to produce Ang II peptide in neonatal rat ventricular myocytes (NRVM). Cells expressing intracellular Ang II were significantly larger than control cells after 96h of infection (3). The addition of extracellular Ang II to the culture media led to an additional increase in cellular protein synthesis. Only extracellular effects of Ang II were blocked by the addition of the ARB losartan to the culture medium (3). These results were reproduced in adult mice, by tail vein injection of an intracellular Ang II expression plasmid, under the control of the α-MHC promoter. The increase in intracellular Ang II expression in the heart caused a significant enlargement of ventricles in these mice, which was not prevented by losartan (3). Since Ang II expression was restricted to the heart, there was no change in blood pressure of these animals. Additionally, intracellular Ang II led to increased mRNA expression of several growth factors including c-jun, IGF-1 and TGF-β in the heart.
These results demonstrated for the first time in vivo, that Ang II plays a critical role as an intracrine hormone in the heart. In cultured cardiac myocytes and fibroblasts, our studies suggested that intracellular Ang II, upregulated the expression of renin, AGT, and AT$_1$, creating a positive feedback loop (77). The latter effect of intracellular Ang II in cardiac fibroblasts was in contrast to the effect of extracellular Ang II in these cells (28, 77). The positive feedback loop might have significant implications in the pathology of chronic diseases, such as cardiac remodeling, that involve persistent activation of the RAS. In cardiac fibroblasts, high glucose-induced intracellular Ang II augmented the production of TGF-$\beta$ and collagen-1. The ARB candesartan partially attenuated, while renin and ACE inhibitors completely prevented, the latter effects of Ang II (76). The lack of inhibition of intracellular effects of Ang II, by ARBs, suggested that either these drugs did not internalize sufficiently or that intracellular Ang II acted independent of the AT$_1$ receptor (4).

**Mechanism of intracellular Ang II actions**

In the above described studies utilizing dialysis or microinjection of Ang II into cardiomyocytes or VSMCs, Ang II actions could be blocked by intracellular, not extracellular, application of an ARB, suggesting involvement of an intracellular AT$_1$-like receptor. In these cells AT$_1$-like binding sites were associated with phospholipase C and protein kinase C activation (33). However, in A7r5 VSMCs and CHO cells, which do not express functionally relevant amounts of AT$_1$ receptor, intracellular Ang II produced growth effects, suggesting the presence of novel intracellular Ang II binding sites (4, 34, 35). Co-expression of Ang II and AT$_1$ receptor in COS-7 and CHO-K1 cells caused greater nuclear localization of the AT$_1$ receptor and stimulated cAMP response element-
binding protein (CREB) activity (13). The latter involved activation of p38 MAPK but not ERK1/2 in A10 VSMCs (12). Recently, it was reported that the effects of intracellular dialysis of Ang II on the L-type inward calcium current in the failing heart of cardiomyopathic hamsters were inhibited by eplerenone, a mineralocorticoid receptor antagonist. Aldosterone reversed the inhibition by eplerenone, suggesting that the aldosterone system also interacts with the intracellular RAS (24). In a yeast two-hybrid screen, intracellular Ang II was reported to bind to mitochondrial proteins of the NADH dehydrogenase complex (14). This latter finding was confirmed in renal proximal tubule cells. The interaction of Ang II with mitochondrial proteins modified oxidative phosphorylation and ROS production (14), which might have contributed to the development of hypertension in transgenic mice expressing intracellular Ang II (65). A recently described non-AT₁, non-AT₂ Ang II binding was identified to be a membrane bound variant of neurolysin (41, 91). Neurolysin is an endopeptidase with wide tissue distribution and cytosolic, mitochondrial, and plasma membrane localization on cells (91). Interestingly, neurolysin is similar to the previously described soluble angiotensin binding protein in the liver (82). The pathophysiological function of these novel Ang II binding proteins remains to be elucidated. Nonetheless, these studies point to plurality of the mechanisms of intracellular Ang II actions, which might depend on the source of Ang II, i.e. internalized or synthesized intracellularly, which in turn might affect the intracellular location and function. Of additional interest are yet unidentified intracellular Ang II binding sites, such as those speculated in A7r5 VSMCs and CHO cells and those described on chromatin (4, 35, 64), identification of which is required for complete understanding of this system and therapeutic modulation in pathological states.
**Intracellular cardiac Ang II synthesis**

Though Ang II internalized via AT₁ receptor could possibly act as an intracrine factor in a pathophysiological setting, *in situ* generation of Ang II would provide a more convincing argument in favor of intracellular effects. As alluded to earlier, the secretory nature of AGT and renin suggests only extracellular synthesis of Ang II. However, observations such as uptake and activation of prorenin by cardiomyocytes, with a resultant increase in intracellular Ang II levels, indicate that intracellular Ang II synthesis might occur following sequestration of prorenin and AGT by these cells (53, 57, 89). Additionally, an intracellular Exon 1A isoform of renin is expressed in rat heart, levels of which are increased 4-fold in the infarcted heart, which could contribute to intracellular Ang II generation (10). The intracellular isoform of renin appears to be active as overexpression of this isoform increases mitochondrial apoptosis in H9c2 cells and increases aldosterone production in transgenic rats (58, 92). Further, studies on renin and AGT dialysis into cardiomyocytes, described above, and those on isolated perfused rat hearts suggested intracellular generation of Ang II (18, 20). In the latter study, the tissue Ang II levels were increased following perfusion of the heart either with renin plus AGT, Ang I, or Ang II. Addition of losartan to the renin plus AGT perfusion had no significant effect, whereas losartan in the perfusions with Ang I or Ang II decreased tissue Ang II to undetectable levels. These observations suggested renin and AGT uptake and intracellular Ang II production in the heart (18). More recently, a newly identified metabolite of AGT, Ang-(1-12), has been reported to be internalized by rat cardiomyocytes and metabolized to bioactive Ang peptides, including Ang II (1). The conversion of Ang-(1-12) into Ang II, which was independent of renin but likely involved
chymase, was significantly enhanced in SHR compared to WKY rats. All of the above studies supported intracellular Ang II production by cardiomyocytes; however, a direct demonstration in a pathophysiological setting was lacking. We performed detailed studies to determine the mechanism and site of Ang II generation in cardiomyocytes and fibroblasts, as described below.

**Mechanism of intracellular Ang II synthesis in cultured cardiomyocytes**

Ang II synthesis in NRVM was stimulated by isoproterenol and a high glucose concentration in serum-free culture medium (80). Changes in Ang II levels in the cell lysate and culture medium were measured separately in the presence or absence of the ARB candesartan. Under these conditions, Ang II would be synthesized from endogenously produced, not internalized RAS components. Further, an increase in Ang II levels in the cell lysate, in the presence of candesartan, would indicate intracellular synthesis, not uptake from the culture medium. Exposure to isoproterenol increased Ang II levels, both in the cell lysate and culture medium, suggesting intracellular synthesis as well as secretion and/or extracellular synthesis of Ang II. In the case of high glucose, Ang II levels were increased only in the cell lysate. Further experiments showed that AGT and renin were retained intracellularly in the presence of high glucose, the reason for which is unknown. Confocal microscopy revealed that isoproterenol-stimulated Ang II was localized mainly along actin filaments, likely in secretory vesicles; whereas Ang II staining was observed in the perinuclear region and inside of nuclei of cardiomyocytes exposed to high glucose. These results indicated that intracellular Ang II was synthesized at different sites in response to isoproterenol and high glucose, likely due to redistribution of RAS components under these conditions.
Using specific enzyme inhibitors, it was observed that isoproterenol-induced Ang II synthesis was catalyzed by renin and ACE; whereas the high-glucose-mediated Ang II generation involved renin and chymase. Similar to NRVM, adult mouse cardiomyocytes showed enhanced intracellular Ang II production when exposed to high glucose medium (Fig. 1). These studies demonstrated that cardiomyocytes synthesized Ang II solely intracellularly in high glucose conditions (80).

**Mechanism of intracellular Ang II synthesis in cultured cardiac fibroblasts**

Fibroblasts constitute the second major cell type in the heart, that have a significant role in cardiac remodeling in various pathological conditions. Fibroblasts have been reported to contain a complete RAS and respond to stimulation with Ang II by extracellular matrix production (69). The close proximity to and interaction with cardiac myocytes make cardiac fibroblasts of critical importance when considering paracrine and intracrine production of angiotensin II in the heart. We performed experiments in these cells similar to those described in cardiomyocytes (76). It was observed that treatment of cardiac fibroblasts with either high glucose or isoproterenol induced both intracellular and extracellular production of Ang II. Ang II production, intracellular and extracellular, was dependent on renin and ACE, not chymase, both with high glucose and isoproterenol treatment. Immunocytochemical analysis showed punctate cytoplasmic and perinuclear staining for Ang II. Taken together, our studies in cardiac myocytes and fibroblasts suggested that the site of Ang II synthesis, i.e. intracellular (secretory vesicles or elsewhere) or extracellular, and the enzymes involved, largely depend on the cell type and the stimulus (Fig. 2).
Stimulation of the intracellular RAS by glucose in other cell types

The most significant observation regarding the intracellular cardiac RAS is the high glucose-induced intracellular synthesis and complete retention of Ang II in cardiomyocytes. These observations are important not only for the understanding of the RAS in diabetes, but also for clinical management in diabetic cardiomyopathy. Interestingly, the conversion of a secretory system to an intracellular system by glucose is not unique to cardiomyocytes. It was reported in rat VSMCs that Ang II production was mainly extracellular and ACE-mediated in normal glucose conditions, becoming largely intracellular and chymase-catalyzed in high glucose medium (50). Podocytes have an important role in the pathogenesis of proteinuria in diabetic nephropathy. Exposure of podocytes to high glucose doubled intracellular Ang II levels, which could be blocked by chymostatin, but not by captopril (29). Other investigators also reported increased Ang II levels in the cell lysate of podocytes exposed to high glucose conditions (94, 95). Similarly, increased intracellular Ang II levels in response to high glucose have been described in renal mesangial and proximal tubular cells (16, 84, 90). These studies suggest that several major cell types produce Ang II intracellularly and that the intracellular RAS is significantly upregulated by high glucose conditions.

The Intracellular RAS in Diabetic Cardiomyopathy

Diabetic cardiomyopathy is defined as heart failure manifested independently of other risk factors, and closely associated with diabetes mellitus (42). The etiology of this disease remains unclear, but may be related to several factors, including abnormal metabolism of glucose in myocardial tissue. Involvement of the RAS in diabetic
cardiomyopathy has been demonstrated by experimental and clinical studies. As discussed above, high glucose upregulated the intracellular RAS in cardiomyocytes (79), cardiac fibroblasts (76), VSMCs (50), and renal mesangial cells (75). Thus, diabetes mellitus provided an important pathological condition to study the role of the intracellular RAS in vivo. Our in vitro studies suggested that in diabetic heart, cardiomyocytes will generate primarily intracellular Ang II, via renin and chymase, whereas fibroblasts will generate both intracellular and extracellular Ang II through renin and ACE. Thus, Ang II will have both intracrine and autocrine/paracrine effects on cardiac cells in diabetic heart. ACE inhibitors will prevent Ang II synthesis only in cardiac fibroblasts. Significantly, in humans, intracellular Ang II levels in cardiac myocytes were 3.4-fold higher in diabetic patients, compared to non-diabetics and an additional 2-fold higher in diabetic hypertensive patients, compared to diabetic non-hypertensive patients (36). Interestingly, these patients were on ACE inhibitor therapy. We have reported previously that ARBs do not abolish intracellular Ang II mediated effects in the heart (3). Therefore, current therapeutic modalities utilizing ACE inhibitors and ARBs may only be partially effective in diabetic cardiomyopathy. The benefits of ACE inhibitors and ARBs during and after MI have been found to be greater in diabetics than non-diabetics and activation of the RAS has been implicated in diabetes (52). However, following MI, the incidence of heart failure and mortality rates are increased two-fold in patients with diabetes, compared to non-diabetics (5, 54). There is a growing consensus that inhibition of the RAS using ARBs and ACE inhibitors has not provided as much cardiovascular benefit as anticipated (86, 93).
We utilized a streptozotocin-induced type-1 model of diabetes in rats to study activation of the cardiac intracellular RAS and the role in cardiac remodeling (81). One week of diabetes significantly increased intracellular Ang II levels in cardiac myocytes isolated from diabetic hearts compared to controls. Intracellular levels of Ang II were not normalized by candesartan, suggesting that Ang II was synthesized intracellularly, not internalized through AT$_1$ receptor. Similar to the in vitro studies discussed previously, an increase in intracellular Ang II levels in cardiac myocytes was prevented by treatment of diabetic rats by aliskiren (renin inhibitor), but not by benazeprilat. Diabetes-induced superoxide production and cardiac fibrosis were partially inhibited by candesartan and benazepril treatment, whereas aliskiren produced complete inhibition (81). These observations suggested that renin inhibition has a more pronounced effect than ARBs and ACE inhibitors, on diabetic complications and may be clinically more efficacious.

We further extended these observations by studying comparative efficacy of RAS inhibition at the level of renin, ACE, and AT$_1$ receptor, on cardiac function in a mouse model of streptozotocin-induced diabetes (70). Diastolic cardiac dysfunction evident at 10 wks of diabetes, measured by mitral annulus flow (E/A ratio), was completely prevented by treatment with all three agents. However, aliskiren resulted in a better E/A ratio at 10 wks compared to 0 day. The increase in isovolumetric relaxation time (IVRT) was completely prevented by aliskiren and benazeprilat, while valsartan was only partially effective. A significant reduction in ejection fraction (EF), fractional shortening (FS), and cardiac output in diabetic animals were prevented by all three drugs. However, aliskiren and benazeprilat treatment showed statistically significant improvement in EF and FS, at 10 wks compared to 0 day. There was no difference in
the mean arterial pressure between control, diabetic, or diabetic groups treated with RAS inhibitors, suggesting that any effect of these drugs on cardiac function was independent of blood pressure. These data showed that renin inhibition provided similar/better cardiac protection compared to ACE inhibitors and ARBs in diabetic cardiomyopathy (70). Protective effects of ACE inhibitors and ARBs may have been partially mediated through non-RAS related mechanisms, such as an effect on the kallikrein-kinin system and PPARγ activation (32, 68). A genetic model, such as AT1 receptor deficient mice, will likely provide better insight on the role of intracellular versus extracellular Ang II in diabetic conditions.

Direct Effect of Ang II in the Heart: Extracellular versus Intracellular Ang II

Multiple in vitro studies have shown that Ang II has direct effect on cell signaling, resulting in hypertrophy in cardiac myocytes and proliferation of cardiac fibroblasts (7, 28, 73). Ang II causes changes in gene expression, resulting in the secretion of growth factors and extracellular matrix proteins. However, direct effects of Ang II on cardiac remodeling are difficult to study in vivo as it is not possible to prevent systemic effects of Ang II, such as hypertension, from affecting cardiac function. Several experimental and genetic models have been utilized to study a direct effect of the RAS in the heart. Results of these studies have not been consistent, which has prompted some investigators to suggest that Ang II alone, independent of blood pressure, does not produce cardiac hypertrophy and fibrosis (66). A requirement of other factors, such as oxidative stress, inflammation, and aldosterone has been proposed for Ang II to produce pathological effects in the heart (49). However, these factors may very well be the product of Ang II actions. These studies utilizing genetic models were designed to
activate the extracellular cardiac RAS. A recent report described a transgenic mouse expressing intracellular Ang II in fusion with a fluorescent protein (65). The fusion protein was detected in several tissues including kidneys and the heart. These mice were hypertensive and developed thrombotic microangiopathy. However, no significant histological changes were observed in the heart, which is in contrast to our observation of significant cardiac hypertrophy in mice injected with an intracellular Ang II expressing vector (3). One of the differences in these two studies was the use of Ang II as a fusion protein versus native peptide. No cardiac histological change in the fusion protein study was particularly noteworthy in view of the elevated blood pressure in these animals. Effects on cardiac function or other parameters such as oxidative stress were not reported. As discussed by DeMello and Frohlich (23), caution should be used when interpreting results of studies utilizing different experimental conditions; and direct but subtle effects of Ang II, including those on cellular communication and metabolism, should not be ignored (23). It will be interesting to study cardiac pathology in intracellular Ang II transgenic mice in the diabetic state, which is associated with upregulation of the endogenous intracellular RAS.

One question that arises is whether intracellular and extracellular Ang II work independently of or in concert with each other. Re has proposed an intracrine hypothesis, a key feature of which is that intracrine factors participate in the formation of cellular memory through positive feedback loops (62). The latter might be important for long-term potentiation of autocrine/paracrine effects of intracrine peptides. As alluded to earlier, extracellular Ang II binding to AT₁ causes internalization of the receptor, causing downregulation of AT₁-mediated signaling. The latter appears to be a protective
mechanism to prevent continuous activation of the system, which otherwise might produce deleterious effects. Regulation of blood pressure and fluid and electrolyte homeostasis likely falls in this category of acute effects of extracellular Ang II. To produce chronic effects such as cardiac hypertrophy, the system needs to be continuously activated. Our studies have suggested that intracellular Ang II is a positive regulator of the RAS, i.e. increases expression of AGT, renin and AT$_1$ receptor, both in cardiomyocytes and fibroblasts (77). Thus, in addition to having independent cellular effects, intracellular Ang II likely keeps AT$_1$ receptor levels sufficiently high for long term activity of extracellular Ang II. Thus, the intra- and extra-cellular RAS work in concert rather than as separate systems. However, the above hypothesis needs to be verified in a suitable *in vivo* model.

**Perspective and Significance**

Several studies have demonstrated effects of intracellular Ang II in multiple cell types. The occurrence of Ang II intracellularly does not solely depend on AT$_1$-mediated internalization; instead all major cell types synthesize Ang II intracellularly under certain pathophysiological conditions (Fig. 2). Hyperglycemia appears to be a common condition that significantly enhances intracellular Ang II generation in several cells. The intracellular site of Ang II synthesis and the participating enzymes may differ between cells. The latter may influence modulation of the RAS in a pathological state, particularly diabetes. From our current understanding of the cardiac intracellular RAS, it appears that therapeutic modalities, such as a renin inhibitor, will provide more complete blockade of the cardiac RAS in diabetes than an ARB or ACE inhibitor. The latter may apply to diabetic nephropathy and retinopathy as well; however, that remains to be
determined. More studies are required to delineate specific roles of intracellular Ang II, independent of and in conjunction with extracellular Ang II. Additionally, the mechanism of intracellular Ang II actions, mediated by AT1-like or other intracellular binding sites, needs to be determined. Knowledge of the intracellular RAS will also enhance our understanding of other peptide hormonal systems which have demonstrated similar intracrine modes of action.

**Acknowledgement**

This work was supported by NIH grant 5R01HL090817.


16. **Cristovam PC, Arnoni CP, de Andrade MC, Casarini DE, Pereira LG, Schor N, and Boim MA.** ACE- and chymase-dependent angiotensin II generation in normal


Figure Legends

Figure 1. Intracellular Ang II in adult mouse cardiac myocytes. Cardiac myocytes were isolated from young adult mice using the Langendorff method. Myocytes were grown in medium containing normal glucose (5.5 mM, NG), high glucose (25 mM), or HG + candesartan (1 µM, Cand). After 24 h, cells were co-immunostained for Ang II (green) and α-sarcomeric actin (red) and visualized with a confocal microscope. A high degree of intracellular Ang II staining (yellow) was observed in HG medium compared to NG. Candesartan did not have any effect on levels of intracellular Ang II, suggesting intracellular synthesis.

Figure 2. Sources of intracellular Ang II in cardiomyocytes. A. Internalization: Ang II-AT$_1$ receptor complex undergoes internalization in the process of cellular signaling. The complex dissociates intracellularly, resulting in the degradation or release of Ang II in the cytoplasm. B. Intracellular synthesis following stimulation with high glucose: The precise intracellular site of Ang II synthesis is not known; however, it appears to occur outside of the secretory pathway. In cardiomyocytes, renin and chymase are involved in intracellular Ang II synthesis. C. Intracellular synthesis following stimulation with isoproterenol: In this case, Ang II synthesis likely occurs inside the secretory vesicles, due to cosorting of AGT, renin, and ACE. Part of the secretory vesicle Ang II content is released into the cytoplasm, while what remains is secreted outside of the cell. Cytoplasmic Ang II can freely diffuse into nucleus due to the small size. Ang II likely has multiple interaction sites in the cytoplasmic organelles and nucleus.