Intracellular Angiotensin II Production in Diabetic Rats is Correlated with Cardiomyocyte Apoptosis, Oxidative Stress, and Cardiac Fibrosis

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ABSTRACT

Objective: Many of the effects of Angiotensin (Ang) II are mediated through specific plasma membrane receptors. However, Ang II also elicits biological effects from the interior of the cell (intracrine), some of which are not inhibited by angiotensin receptor blockers (ARBs). Recent in vitro studies, have identified high glucose as a potent stimulus for the intracellular synthesis of Ang II, the production of which is mainly chymase dependent. In the present study, we determined whether hyperglycemia activates the cardiac intracellular renin-angiotensin system (RAS) in vivo and whether ARBs, ACE, or renin inhibitors block synthesis and effects of intracellular Ang II (iAng II).

Research Design and Methods: Diabetes was induced in adult male rats by streptozotocin. Diabetic rats were treated with insulin, candesartan (ARB), benazepril (ACE inhibitor), or aliskiren (renin inhibitor).

Results: One week of diabetes significantly increased iAng II levels in cardiac myocytes, which were not normalized by candesartan, suggesting that Ang II was synthesized intracellularly, not internalized through AT1 receptor. Increased intracellular levels of Ang II, angiotensinogen (AGT) and renin were observed by confocal microscopy. iAng II synthesis was blocked by aliskiren; but, not benazepril. Diabetes-induced superoxide production and cardiac fibrosis were partially inhibited by candesartan and benazepril, whereas aliskiren produced complete inhibition. Myocyte apoptosis was partially inhibited by all three agents.

Conclusions: Diabetes activates the cardiac intracellular RAS, which increases oxidative stress and cardiac fibrosis. Renin inhibition has a more pronounced effect than ARBs and ACE inhibitors on these diabetic complications and may be clinically more efficacious.
Involvement of the renin-angiotensin system (RAS) in human pathophysiology, has expanded to include several diseases, beyond a traditional role in salt-water homeostasis (1). In diabetes, there is significant over-activity of the RAS, which is reversed by treatment with RAS inhibitors, thus decreasing diabetic complications (2). Activation of the RAS in diabetes includes activation of new components, such as the pro(renin) receptor (3) and Angiotensin (Ang) II-independent effects, mediated through interaction of pro(renin), with the pro(renin) receptor (4). While circulating renin and Ang II levels are reduced in diabetes, prorenin levels are enhanced several fold (5, 6). Prorenin may have dual effects, providing for generation of Ang I at tissue sites, through receptor-mediated non-proteolytic activation; and directly through activation of receptor-mediated signaling pathways (4, 7, 8). Ang II-independent RAS actions suggest that efficacy of RAS inhibitors, angiotensin receptor blockers (ARBs) and ACE inhibitors, would have limitations in hyperglycemic conditions. Recent meta-analyses of clinical trials have suggested that currently used RAS blockers, may not provide additional benefits in diabetic patients, compared to non-diabetics (9, 10).

We recently reported a novel aspect of the RAS, the intracellular RAS, having identified an intracellular or intracrine system (11, 12). In cardiac myocytes and fibroblasts, we demonstrated the presence of RAS components and synthesis of Ang II intracellularly (13, 14). Hyperglycemia selectively upregulates the intracellular system in cardiac myocytes, vascular smooth muscle cells (VSMC), and renal mesangial cells, where Ang II synthesis is largely catalyzed by chymase, not ACE (14-18). We and others have previously reported that intracellular Ang II (iAng II) elicits biological effects, some of which are not blocked by ARBs (19-22). These observations further support the speculation that currently available RAS inhibitors may not provide the anticipated cardiovascular benefits in diabetic conditions (23). In this study, we have examined the activation of the cardiac intracellular RAS in a rat model of diabetes. We also determined the role of iAng II in diabetes-induced oxidative stress, cardiac myocyte apoptosis and cardiac fibrosis and the efficacy of different RAS blockers under hyperglycemic conditions.

**RESEARCH DESIGN AND METHODS**

All animal use was approved by the Institutional Animal Care and Use Committee of the Texas A&M Health Science Center. The AT1 receptor blocker, candesartan, was obtained from AstraZeneca (Wilmington, DE); the renin inhibitor, aliskiren, was from Novartis (Cambridge, MA); the ACE inhibitor, benazepril, was from Sigma; and insulin (Humulin N) was from Eli Lilly (Indianapolis, IN).

**Induction of diabetes and treatment of animals.** Diabetes was induced by a single injection of streptozotocin (STZ, 65 mg/kg body weight, IP) dissolved in 0.1 M sodium citrate buffered saline (pH 4.5), in adult male Sprague Dawley rats (250-300 g). Control animals received buffered saline alone. Diabetes was confirmed by sustained blood glucose levels >15 mmol/L, as determined 48 h after STZ injection and on alternate days thereafter. Diabetic rats, in groups of nine animals,
were treated with either insulin (2-5 U, BID, SC), candesartan (1 mg/kg, IP), aliskiren (30 mg/kg, oral), or benazepril (10 mg/kg, oral), daily for 7 days, beginning 48 h after STZ injection. Twenty-four hours following the last treatment, animals were weighed, anesthetized using ketamine/xylazine (50/5 mg/kg) and hearts were isolated and weighed before perfusion, the latter using the Langendorff methodology.

**Isolation of cardiac myocytes and measurement of iAng II.** Hearts were isolated and perfused with Krebs-Henseleit bicarbonate buffer, followed by digestion with collagenase II (0.1 % w/v). Myocytes were separated from non-myocytes by differential centrifugation at 25 X g. The purity of the myocyte preparations using this procedure was >90%, as analyzed by FACS, using anti-sarcomeric myosin (MF-20) and anti-sarcomeric actin antibody. The pellet containing myocytes was processed for Ang II extraction, as described previously (14). Briefly, cells were lysed in ice-cold 1 M acetic acid, containing a protease inhibitor cocktail (Sigma), by brief sonication. The lysate was sedimented at 20,000 X g for 10 min and the supernatant dried in a vacufuge, followed by reconstitution in 1% acetic acid. The samples were applied to a conditioned DSC-18 column (Supelco), washed, and eluted with methanol. The eluted samples were dried and reconstituted in PBS for ELISA. For isolation of Ang II from plasma, an equal volume of 2% acetic acid was added to plasma, followed by filtration through Amicon Ultra-15 filters. The filtrate was applied to DSC-18 columns and Ang II was eluted, as described for the cell lysates. Using the above procedure, we have obtained >90% recovery of exogenously added Ang II. Ang II was measured by quantitative, competitive ELISA, using a specific anti-Ang II antibody (Peninsula Labs), which was previously validated by HPLC-chip/mass spectrometric analysis (14). ELISA was performed on protein-A and anti-Ang II antibody-coated 96-well dishes. Competitive binding of synthetic biotinylated Ang II, in the presence of the extracted peptide, was detected with streptavidin-horseradish peroxidase conjugate. A standard curve, generated from binding of a constant amount of biotinylated Ang II with increasing concentrations of non-biotinylated synthetic Ang II, was used to calculate the concentration of the peptide in the sample. The concentration of Ang II in the cell lysates is expressed as fmoles per mg protein and in plasma as fmoles per mL.

**Immunohistochemistry of RAS components.** Hearts were frozen in OCT compound (Tissue-Tek, Sakura Finetek, USA), at -80 °C, for immunofluorescence staining of Ang II, renin, and AGT. Frozen tissue was cut into 5 µm sections, which were air-dried, fixed with 4 % formaldehyde and permeabilized using 0.2 % Triton-X100. Nonspecific binding was blocked by 5 % BSA, for 1 hour, at room temperature. The sections were incubated with either anti-Ang II antibody (1:100, Peninsula Labs), anti-renin antibody (1:100, gift from Dr. Tadashi Inagami, Vanderbilt University), or anti-angiotensinogen (AGT) antibody (1:500, Swant). The sections were co-stained for α-sarcomeric actin and laminin, where indicated. After washings, the sections were incubated with respective secondary antibodies. Specificity of the staining was determined by pre-adsorption of primary antibodies with the antigen or by using secondary antibody alone. Images were
acquired with a confocal fluorescence microscope (Olympus Fluoview 300). Fluorescence intensities in tissue sections were determined by digital microscopy software (Slide Book 4.2), after subtracting background fluorescence.

**Cardiac myocyte apoptosis.** Apoptotic cardiac myocytes were detected in paraffin-embedded heart sections using the terminal deoxynucleotide transferase-mediated dUTP nick end labeling (TUNEL) assay and cleaved caspase-3 staining. TUNEL assay was performed using an assay kit (Millipore Corporation, Temecula, CA), as per the manufacturer’s instructions. Cytoplasm and nuclei from the myocytes were counter-stained using α-sarcomeric actin antibody and DAPI, respectively. For cleaved caspase-3 staining, deparaffinized sections were subjected to antigen retrieval in 0.01 M citrate buffer (pH 6.0), by microwaving. After blocking with 5% BSA, the sections were incubated with rabbit monoclonal anti–cleaved caspase-3 antibody (1:200; Cell Signaling Technology, Danvers, MA) overnight at 4 °C, followed by fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (1:200; Molecular Probes). The number of positively stained nuclei were counted from 20 fields/heart (about 25,000 cells) and three hearts/treatment group.

**Reactive oxygen species (ROS) detection in the heart.** Superoxide production in the hearts was detected by dihydroethidium staining (DHE, Sigma-Aldrich). Frozen heart sections (20 µm thick) were incubated with 10 µM DHE, at 37 °C for 45 min, in a humidified chamber protected from light. Fluorescent images, obtained with an Olympus FV300 confocal microscope, were analyzed with Slide Book 4.2. The mean DHE fluorescence intensity of myocyte nuclei was calculated by dividing the combined fluorescence value of the pixels by the total number of pixels, in fifteen randomly selected fields observed with identical laser and photomultiplier settings.

**Cardiac Fibrosis.** Cardiac interstitial fibrosis was determined by Masson’s trichrome staining on 5 µm paraffin-embedded sections. The extent and degree of fibrosis was subjectively graded on a scale of 0-4. Grade 0 signified no apparent collagen fiber proliferation except for small islets of fibrous tissue around the capillaries, as well as an intercellular single layer of collagenous tissue, as in normal myocardium. Focal and minimal fibrosis was graded as 1, mild patchy fibrosis as grade 2, moderate diffuse fibrosis as grade 3 and the most prominent fibrosis, covering a major area of the specimen, was classified as 4. A minimum of three sections per heart, with five fields per section, and three animals per experimental group, were analyzed and results presented as an average grade.

**Statistical analysis.** Values are expressed as the means ± SE. ANOVA with Tukey’s post hoc test was used for statistical analysis. *P* <0.05 was considered statistically significant.

**RESULTS**

*Hyperglycemia increases intracellular levels of Ang II in cardiac myocytes from diabetic heart.* Diabetes was induced in adult male rats by STZ injection. One group of diabetic animals was treated with insulin to confirm that the observed effects in the experimental groups were secondary to hyperglycemia. One week of diabetes significantly reduced body and heart weights, which were normalized by insulin treatment, but
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not by any of the RAS inhibitors (Table 1). However, no significant effect on the heart weight/body weight ratio (Table 1) or plasma Ang II levels (Fig. 1B) was observed, which is consistent with previous reports (24, 25). Ang II levels in cardiac myocytes, which were isolated after perfusion of the hearts and enzymatic dispersion, represented Ang II present intracellularly. To determine the source of iAng II, i.e., intracellular synthesis or AT$_1$-mediated internalization, one group of diabetic animals was treated with the AT$_1$ antagonist candesartan to prevent receptor-mediated uptake. As shown in Fig. 1a, cardiac myocytes from diabetic rat hearts demonstrated a 9.4-fold elevation in the levels of iAng II (183 ± 13 fmol/mg protein), compared to cells from control animals (19 ± 4 fmol/mg protein). Normalization of blood glucose levels by insulin, in STZ-treated rats, completely blocked the rise in iAng II levels, indicating that the latter was a specific effect of hyperglycemia.

Treatment of diabetic rats with candesartan, partially reduced iAng II levels, suggesting the major source of iAng II was intracellular synthesis, which is consistent with our previous report in neonatal rat ventricular myocytes (NRVM) (14). Intracellular synthesis of Ang II is not blocked by an ACE inhibitor. We and others had previously reported that several cell types (NRVM, VSMCs, and renal mesangial cells) utilize alternative pathways to synthesize Ang II in high glucose culture conditions (14-18). To determine the mechanism of hyperglycemia-induced cardiac iAng II synthesis in vivo, diabetic rats were treated with either a renin inhibitor (aliskiren) or an ACE-inhibitor (benazepril). As shown in Fig. 1A, aliskiren completely normalized (33 ± 7 fmol/mg protein) iAng II levels in diabetic rat cardiac myocytes, while benazepril did not have any effect (143 ± 8 fmol/mg protein). These results indicated that the observed increase in iAng II was not catalyzed by ACE, which is consistent with intracellular synthesis of Ang II. None of the RAS blocking drugs significantly altered plasma Ang II levels, which were measured 24 h after the last dose. The latter observation corroborated with the reported 2 - 6 h period for reactive changes in plasma levels of Ang II, which return to baseline between 14 - 30 h after drug intake (26-28).

Immunohistochemical localization of Ang II in rat heart. To further confirm elevation of Ang II in cardiac myocytes and intracellular localization, frozen heart sections were immunostained with anti-Ang II antibody and visualized using confocal microscopy. Sections were counterstained for laminin to mark cell boundaries (peripheral green/yellow staining in merged images), α-sarcomeric actin to identify cardiac myocytes (red), and DAPI to identify nuclei (blue). Significantly increased levels of Ang II staining, which co-localized with α-sarcomeric actin, were observed in diabetic rat hearts (Fig. 1 C-F). Quantification of fluorescence intensity revealed about a 5-fold increase in iAng II levels (Fig. 1G), consistent with the Ang II measurement by ELISA (Fig. 1A). Immunohistochemistry also confirmed that candesartan partially reduced iAng II levels, benazepril did not have any effect and aliskiren completely prevented the
Intracellular Ang II in diabetes (Fig. 1G).

**Intracellular localization of AGT and renin in cardiac myocytes.** Intracellular synthesis of Ang II would require the presence of the precursor molecule AGT and processing enzyme, renin, intracellularly. To demonstrate this possibility, immunohistochemistry was performed on heart sections using anti-AGT and anti-renin antibodies, along with counter-staining for α-sarcomeric actin, as described previously (24). Elevated levels of AGT and renin were apparent in cardiac myocytes of diabetic hearts (green/yellow staining), compared to control, confirming activation of the intracellular RAS in hyperglycemic conditions (Fig. 2).

**Intracellular Ang II is correlated with hyperglycemia-induced oxidative stress.** Hyperglycemia is known to induce myocardial oxidative stress, which may be related to glucose metabolism or activation of cytokines and other hormones. To determine if there was a role of iAng II, superoxide production was detected by DHE staining in frozen heart sections of diabetic rats, treated with different RAS inhibitors. As shown in Fig. 3, diabetic hearts showed enhanced superoxide production, which was prevented in insulin treated animals. Treatment of diabetic rats with candesartan or benazepril, significantly, but not completely, reduced oxidative stress, while aliskiren blocked completely. Our previous studies had indicated that ARBs and ACE-inhibitors were ineffective in blocking the intracellular RAS, unlike a renin inhibitor that blocks both the intracellular and extracellular systems (14, 19, 22). The observed partial efficacy of candesartan and benazepril, strongly suggested that iAng II contributed to hyperglycemia-induced oxidative stress in the myocardium.

**Intracellular Ang II is correlated with hyperglycemia-induced cardiac myocyte apoptosis.** Cardiac myocyte apoptosis was determined by TUNEL assay and activated caspase-3 immunostaining. Fig. 4 shows an increased number of apoptotic cells in the heart sections. Quantification of apoptotic cells (Fig. 4 C and F) showed a 5 to 8-fold increase in diabetic hearts, compared to control, by both TUNEL assay and caspase-3 staining. Normalization of blood glucose by insulin or blockade of the RAS with the three different inhibitors, significantly reduced the number of apoptotic cells, but did not prevent apoptosis completely. There was a significant difference between aliskiren and benazepril treated animals, with aliskiren being more protective.

**Intracellular Ang II is correlated with hyperglycemia-induced cardiac fibrosis.** Cardiac fibrosis is an important pathogenic factor in diabetes-induced diastolic dysfunction. Paraffin-embedded heart sections were stained with Masson’s Trichrome and the degree of blue staining evaluated on a scale of 0-4, as described in Research Design and Methods. Even after only one week of diabetes, the overall staining for fibrosis was enhanced in hearts from diabetic rats (grade 1.5), compared to control animals (grade 0) (Fig. 5). Insulin treatment completely prevented the increase in fibrosis (grade 0.04). Candesartan and benazepril reduced the degree of fibrosis (grade 0.43 and 0.88, respectively), whereas aliskiren had a more pronounced reduction of fibrosis (grade 0.25) in diabetic rat hearts (Fig. 5).
DISCUSSION

In this study, we observe a dramatic activation of the intracellular RAS, a novel aspect of the tissue RAS, in diabetic rat hearts. We also demonstrate that iAng II is correlated with the development of pathological conditions associated with diabetes. Significantly, we observed that blockade of the RAS by a renin inhibitor, in diabetic rats, provided greater protection from oxidative stress and cardiac fibrosis, compared to inhibition with an AT1 antagonist or ACE inhibitor.

We first described a physiologically relevant intracellular RAS in NRVM (14), defined as the presence of the precursor protein and enzymes, synthesis of Ang II inside the cell, and which was coupled to a biological action (11). The regulation and separation of the intracellular from the extracellular RAS, becomes very obvious in hyperglycemic conditions. High glucose promoted accumulation of AGT, renin, and Ang II intracellularly, resulting in a dramatic rise in iAng II concentrations, without affecting extracellular Ang II levels (14). Similar to NRVM, the intracellular accumulation of RAS components and iAng II synthesis, have also been described in VSM and renal mesangial cells, in high glucose culture conditions (15, 17, 29). To extend the in vitro observations, we determined whether a similar activation of the intracellular RAS occurs in adult diabetic animals. We observed a significant increase in intracellular levels of AGT and renin and iAng II synthesis in diabetic rat hearts, as determined by ELISA and confocal immunocytochemistry. In a previous human study, a 3-fold increase in Ang II staining was described in hearts from diabetic patients, compared to non-diabetics, which was enhanced an additional 2.5-fold in diabetic hypertensive patients (30). However, in that study it was not clear if the increased Ang II staining represented activation of an intracellular RAS or was due to internalization of extracellularly synthesized Ang II. We observed an increase in iAng II levels even in the presence of AT1 blockade with candesartan, which strongly supported activation of the intracellular RAS. The latter conclusion was further strengthened by an observed increase in intracellular staining for renin and AGT.

The observation of elevated Ang II levels after removal of the high glucose stimulus, suggested that iAng II was highly stable. The reported half-life of Ang II in the heart is 15 min in vivo and 30 min ex vivo (31). In this regard, it is important to make a distinction between Ang II that was internalized versus that which was synthesized intracellularly, as the intracellular location is likely to be different, which could substantially affect the half-life. The half-life reported in the literature was for Ang II that was internalized through AT1 receptor (31), a major part of which was likely targeted to lysosomes for degradation (32). We measured Ang II that was synthesized intracellularly, which is most likely to occur in organelles or at sites that are not associated with protein degradation.

An interesting and therapeutically significant aspect of the intracellular RAS is that high glucose-induced iAng II synthesis in cardiac myocytes is catalyzed by chymase, not ACE. Chymase levels are significantly elevated in NRVM following exposure to high glucose, while ACE levels remain unchanged (14). Similarly, no change in gene expression of ACE was observed in diabetic rat hearts (33). In the above-referenced human study (30), diabetic
patients who had elevated iAng II levels, were on ACE-inhibitor therapy. In the latter case, a rise in Ang II levels could be attributed to an ‘ACE-escape’ phenomenon, which is believed to occur following prolonged treatment with ACE inhibitors (34). In the present study, the lack of reduction in iAng II levels, following only one week of ACE-inhibitor treatment, strongly suggests an ACE-independent mechanism of iAng II synthesis, corroborating in vitro observations. Upregulation of vascular chymase in diabetic patients and chymase-mediated Ang II generation, in human and rat VSMC and human mesangial cells, has been previously reported (15, 17, 18, 35). Involvement of chymase further strengthens the concept of an intracellular RAS in diabetes.

Renin inhibition by aliskiren completely prevented hyperglycemia-induced iAng II synthesis. The source of renin in the heart has been an issue of debate (36). Circulating prorenin levels are elevated several fold in diabetes, which might also contribute to cardiac levels of pro(renin) (37). Cardiac myocytes have been shown to internalize and activate prorenin, which could contribute to iAng II synthesis (38, 39). In addition, several reports have described expression of renin by cardiac myocytes, fibroblasts, and cardiac mast cells (13, 40-42). Adult rat cardiac myocytes have been shown to express an intracellular form of prorenin, which lacked a portion of the pre-profragment, eliminating the need for proteolytic activation (43). We have previously described a significant increase in intracellular renin levels in NRVM exposed to high glucose conditions (14). In the current study, we observed enhanced staining for renin in the heart of diabetic rats. Thus, inhibition of cardiac iAng II synthesis by aliskiren was consistent with these observations.

Aliskiren, though the most potent inhibitor of human renin (IC50 = 0.6 nM), inhibits rat renin at higher concentrations (IC50 = 80 nM) (44). We chose an aliskiren dose of 30 mg/kg per day based on significant blood pressure lowering effects of this dose in spontaneously hypertensive rats (45). Pharmacokinetic studies of aliskiren in Sprague-Dawley rats (species used in this manuscript) demonstrated that an oral dose of 30 mg/kg resulted in an AUC (area under the curve) of 3.06 ± 1.8 µM x h, indicating sufficient drug in the bloodstream (45). Several additional characteristics of aliskiren could explain the observed effects in diabetic rats. These include cellular uptake, accumulation upon multiple dosing, longer half-life, and rapid binding to renin with slow dissociation (46). We observed that neonatal rat cardiac myocytes internalized aliskiren in a concentration dependent manner, levels of which were measured at 1, 24, and 48 h following addition to the culture medium. Maximum intracellular levels of aliskiren were observed at 24 h, which were not reduced even after 48 h (data not shown). Aliskiren levels were measured by liquid chromatography tandem mass spectrometry, as previously described (47). Similarly, rats that were treated with aliskiren, at a dose of 10 mg/kg/day for 2 weeks, showed a kidney/plasma ratio of aliskiren in the range of 45 - 64 (47). The latter indicated extensive partitioning of aliskiren to the kidneys, which localized to glomeruli and the walls of small cortical arteries. Persistent renal protective effects of aliskiren, after discontinuation of treatment, also suggests slow clearance and accumulation in tissues (48).
above findings suggest similar partitioning of aliskiren in the heart, which might have resulted in sufficiently high intracellular levels of aliskiren to inhibit rat renin in our studies. Further, tissue accumulation of aliskiren may result in effects on target organs, even at doses which do not affect blood pressure. This was evident from a recent study wherein 2.5 mg/kg aliskiren did not produce a statistically significant sustained reduction in blood-pressure; but, reduced atherosclerotic lesion size significantly, in hypercholesterolemic Ldlr−/− mice (49). Higher doses of aliskiren (up to 50 mg/kg) produced similar results. In the double human renin-AGT transgenic rat model, a dose (of aliskiren) of 0.03 mg/kg, which did not decrease blood pressure, reduced albuminuria and cardiac hypertrophy (50).

iAng II has been shown to produce multiple biological actions, including cardiac hypertrophy (11, 19). Many of the reported iAng II effects are not prevented by ARBs, either due to limited cell permeability of these drugs or to an AT1-independent mechanism of iAng II-mediated effects. We previously demonstrated that iAng II-induced NRVM cell growth and cardiac hypertrophy was not inhibited by ARBs (19). Proliferation of Chinese Hamster Ovary cells, which are deficient in AT1 receptor, demonstrated that some of the effects of iAng II do not require AT1 receptor (22). In addition, enhanced TGF-β/smad signaling was reported in kidneys from diabetic, AT1-knockout mice (51). These findings, together with the observation that iAng II synthesis is chymase-dependent, suggest that ARBs and ACE inhibitors do not block the intracellular RAS, which is activated in diabetes. A renin inhibitor prevents both intracellular and extracellular Ang II synthesis (14), and thus, may prove more beneficial in diabetic conditions. Consistent with the latter hypothesis, we observed that aliskiren was more effective in preventing oxidative stress and cardiac fibrosis, compared to candesartan or benazepril. Effects of aliskiren were unlikely to be mediated through Ang II-independent mechanisms, as aliskiren does not inhibit renin binding to the (pro)renin receptor and ERK activation (8, 47). The effect of these drugs on cardiac myocyte apoptosis, demonstrated that aliskiren was significantly more beneficial than benazepril.

Reversal of cardiac effects in diabetic animals by insulin treatment, indicates that the observed effects were due to hyperglycemia. Though STZ-induced diabetes is representative of Type-1 diabetes, we would also predict activation of the intracellular RAS in Type-2 diabetes. This is based on our unpublished observations of no effect of insulin treatment on high glucose-induced iAng II synthesis in NRVM and patients with type-2 diabetes, who showed enhanced iAng II staining in the heart (30).

As depicted in Fig. 6, activation of the RAS appears to be a major event in hyperglycemia, as a result of increased oxidative stress, increased PKC levels, and/or increased activity of the hexosamine biosynthesis pathway. iAng II could directly produce oxidative stress and cellular apoptosis through unidentified mechanisms and/or could enhance expression of RAS components through a positive feedback mechanism (Unpublished data), resulting in enhanced extracellular Ang II levels as well, particularly via cardiac fibroblasts (13). Extracellular Ang II in turn causes oxidative stress, cardiac myocyte
apoptosis, and cardiac fibrosis through the AT$_1$ receptor. Interrupting this cycle by blocking Ang II synthesis, protects from hyperglycemia induced pathological events. ACE inhibitors or ARBs would block only the extracellular synthesis or actions, respectively, of Ang II; while a renin inhibitor would block both intra- and extracellular Ang II synthesis, the latter providing an explanation for the more pronounced effects of aliskiren observed in this study. In diabetic heart, the source and target of Ang II could be represented by multiple cell types. In addition to NRVM, we previously demonstrated that cardiac fibroblasts respond to high glucose with enhanced activity of the RAS and extracellular matrix production (13). Cardiac fibroblasts increase both intracellular and extracellular Ang II, in contrast to cardiac myocytes, which demonstrate only increased iAng II in high glucose conditions. Additionally, Ang II synthesis by cardiac fibroblasts, extracellular as well as intracellular, is catalyzed by ACE (13). Thus, ACE inhibitors would block Ang II synthesis by cardiac fibroblasts and ARBs would block autocrine/paracrine effects of extracellular Ang II. This explains the partial effects of these agents in diabetic rats. However, these agents would not block high glucose-stimulated iAng II synthesis or intracellular actions in cardiac myocytes. In addition to a direct effect on cardiac myocytes, iAng II synthesized in cardiac myocytes would likely have indirect functional effects on other cells, by stimulating synthesis and release of growth factors and cytokines from myocytes (52). Thus, the intracellular RAS possibly explains progression from microalbuminuria to proteinuria in diabetic patients on ACE inhibitor therapy, the mechanism of resistance to antihypertensive therapy in type 2 diabetes and higher cardiovascular morbidity and mortality in hypertensive patients with diabetes (10, 53-55). Though ARBs and ACE inhibitors would provide some protection to the cardiovascular system through positive hemodynamic effects, partial inhibition of the local RAS, or other non-RAS related mechanisms, such as an effect on PPAR-γ and the kallikrein-kinin system (56-58); additional blockade of the intracellular RAS, using agents such as a renin inhibitor, might prove more beneficial in diabetes. Long-term studies that include cardiac functional analysis will be necessary to validate the above hypothesis.

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**Table 1: Effect of diabetes on body and heart weight**

<table>
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<th>Control</th>
<th>Diabetic</th>
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<tr>
<td></td>
<td>No treatment</td>
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<tr>
<td><strong>BW (g)</strong></td>
<td>337 ± 10</td>
<td>263 ± 8.3*</td>
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<tr>
<td><strong>HW (mg)</strong></td>
<td>1168 ± 54</td>
<td>933 ± 33*</td>
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<td><strong>HW/BW (mg/g)</strong></td>
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Values are expressed as the mean ± SE (N=9), following one week of induction of diabetes. *P < 0.05 vs. control
**FIG. 1.** Ang II levels in cardiac myocytes and plasma. Ang II was measured by a competitive ELISA in cardiac myocytes (A) and plasma (B) of control (Cont), diabetic (STZ), and diabetic rats treated with either insulin (Ins), aliskiren (Alsk), candesartan (Cand), or benazepril (Bnz). Values are expressed as the mean ± SE, N=6. C-F: Intracellular localization of Ang II (yellow dots, indicated by white arrow), as determined by confocal immunofluorescence microscopy, in heart sections from control (C), diabetic (D), diabetic rats treated with aliskiren (E), and diabetic rats treated with candesartan (F). Myocyte profiles were identified by co-staining with α-sarcomeric actin (red) and laminin (yellow, peripheral staining). The blue color indicates nuclear staining by DAPI. Magnification: X1200. G: Quantitative representation of Ang II fluorescence intensity in heart sections (from 5 images/heart, 3 hearts/group). Values are expressed as the mean ± SE, N=15. *p < 0.05 vs. control, †p < 0.05 vs. diabetic rats without any treatment.
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**A**

Myocytes

- Cont
- Ins
- Alsk Cand Bnz
- STZ

**B**

Plasma

- Cont
- Ins
- Alsk Cand Bnz
- STZ

**C**

Heart Sections

- Cont
- Ins
- Alsk Cand Bnz
- STZ

**C, D, E, F**

Images showing cellular structures under different conditions.
FIG. 2. Representative confocal immunofluorescence images of angiotensinogen and renin staining in hearts from control and diabetic rats. Pictures shown are merged images of staining for α-sarcomeric actin (red), laminin (peripheral red staining in upper panel), angiotensinogen (upper panel) or renin (lower panel) (green and yellow staining), and nuclei (blue). Magnification: X900.
FIG. 3. Measurement of oxidative stress in heart sections by DHE staining. These are representative images of DHE-stained heart sections from control (A), diabetic (B), and diabetic rats treated with either insulin (C), aliskiren (D), candesartan (E), or benazepril (F). Magnification: X 60. G: DHE fluorescence intensity was calculated from 5 images/heart, 3 hearts/group. Values are expressed as the mean ± SE (N=15). *p < 0.05 vs. control, †p < 0.05 vs. diabetic rats without any treatment.
FIG. 4. Detection of apoptosis in cardiac myocytes by TUNEL assay and cleaved caspase-3 staining. A and B: TUNEL assay on heart sections from control (A) and diabetic (B) rats. D and E: Staining for cleaved caspase-3 in the hearts of control (D) and diabetic (E) rats. C and F: Quantification of TUNEL (C) and cleaved caspase-3 (F) positive cells (about 25,000 cells were counted in each case). Values are expressed as the mean ± SE. *p < 0.05 vs. control, †p < 0.05 vs. diabetic rats without any treatment.
FIG. 5. Detection of cardiac fibrosis by Masson’s Trichrome staining in heart sections from control (A) diabetic (B) diabetic rats treated with either insulin (C) aliskiren (D) candesartan(E) or benazepril (F). Representative images of 5 sections/heart, 3 hearts/group, observed with a X40 objective.
Fig. 6. Schematic representation of the relationship between hyperglycemia, iAng II, and pathological effects. In hyperglycemia, there is an increase in glucose oxidation through the TCA cycle in mitochondria, which results in enhanced generation of reactive oxygen species. Overproduction of superoxide inhibits glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity, resulting in an accumulation of upstream metabolites of the glycolytic pathway. Increased levels of glyceraldehyde-3-phosphate (GAD-3P) causes activation of PKC isoforms through diacylglycerol (DAG) production and synthesis of advanced glycation end products (AGEs). There is increased shuttling of glucose through the hexosamine biosynthesis pathway, resulting in the modification of transcription factors through o-glycosylation. All these products of hyperglycemia, i.e., oxidative stress, AGEs, PKC, and o-glycosylation of transcription factors activate expression of renin-angiotensin system components. Cardiac myocytes synthesize and retain Ang II intracellularly in hyperglycemia, while cardiac fibroblasts increase both intra- and extracellular Ang II. iAng II could directly increase oxidative stress and cellular apoptosis through unidentified mechanisms and/or could enhance expression of RAS components through a positive feedback mechanism, resulting in enhanced extracellular Ang II levels as well, particularly via cardiac fibroblasts. Extracellular Ang II in turn causes oxidative stress, cardiac myocyte apoptosis, and cardiac fibrosis through the AT1 receptor. Interrupting this cycle by blocking Ang II synthesis, provides protection from hyperglycemia induced pathological events. ACE inhibitors or ARBs would block only the extracellular synthesis or actions, respectively, of Ang II; while a renin inhibitor would block both intra- and extracellular Ang II synthesis, the latter providing an explanation for the more pronounced effects of aliskiren observed in this study.