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

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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 Ang 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-Ang 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 AT_1 receptor. Increased intracellular levels of Ang II, angiotensinogen, and renin were observed by confocal microscopy. iAng II synthesis was blocked by aliskiren but not by 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 diabetes complications and may be clinically more efficacious. ***Diabetes*** **57:3297–3306, 2008**

Involvement of the renin-angiotensin (Ang) system (RAS) in human pathophysiology has expanded to include several diseases beyond a traditional role in saltwater homeostasis (1). In diabetes, there is significant overactivity of the RAS, which is reversed by treatment with RAS inhibitors, thus decreasing diabetes complications (2). Activation of the RAS in diabetes includes activation of new components, such as the pro(renin) receptor (3), and Ang II–independent effects, mediated through interaction of pro(renin), with the pro(renin) receptor (4). Although circulating renin and Ang II levels are reduced in diabetes, prorenin levels are enhanced severalfold (5,6). Prorenin may have dual effects, providing for generation of Ang I at tissue sites through receptor-mediated nonproteolytic activation and directly through activation of receptor-mediated signaling pathways (4,7,8). Ang II–independent RAS actions suggest that efficacy of RAS inhibitors, Ang 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 compared with nondiabetic patients (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 (VSMCs), 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 AT_1 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 wt i.p.) dissolved in 0.1 mol/l 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 insulin (2–5 units s.c., twice daily), candesartan (1 mg/kg i.p.), aliskiren (30 mg/kg orally), or benazepril (10 mg/kg orally) daily for 7 days beginning 48 h after STZ injection. Twenty-four hours after the last treatment, animals were weighed and anesthetized using

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TABLE 1
Effect of diabetes on body and heart weight

		Diabetic				
	Control	No treatment	Insulin	Aliskiren	Candesartan	Benazepril
Body weight (g)	337 ± 10	263 ± 8.3*	307 ± 4.7	248 ± 8.5*	285 ± 5.9*	267 ± 6.9*
Heart weight (mg)	1,168 ± 54	933 ± 33*	1,074 ± 27	867 ± 37*	988 ± 15*	901 ± 16*
Heart weight/body weight (mg/g)	3.46 ± 0.1	3.55 ± 0.1	3.49 ± 0.1	3.50 ± 0.1	3.47 ± 0.1	3.37 ± 0.1

Data are means ± SE ($n = 9$) after 1 week of induction of diabetes. *$P < 0.05$ vs. control.

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 0.1% (wt/vol) collagenase II. Myocytes were separated from nonmyocytes by differential centrifugation at 25*g*. The purity of the myocyte preparations using this procedure was >90%, as analyzed by fluorescence-activated cell sorting, 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 mol/l acetic acid containing a protease inhibitor cocktail (Sigma) by brief sonication. The lysate was sedimented at 20,000*g* for 10 min, and the supernatant was 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 enzyme-linked immunosorbent assay (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 high-performance liquid chromatography–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 nonbiotinylated 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 femtomoles per milligram protein and in plasma as femtomoles per milliliter.

Immunohistochemistry of RAS components. Hearts were frozen in OCT compound (Tissue-Tek; Sakura Finetek) at −80°C for immunofluorescence staining of Ang II, renin, and anti-angiotensinogen (AGT). Frozen tissue was cut into 5-μm sections, which were air-dried, fixed with 4% formaldehyde, and permeabilized using 0.2% Triton X-100. Nonspecific binding was blocked by 5% BSA for 1 h at room temperature. The sections were incubated with anti–Ang II antibody (1:100; Peninsula Labs), anti-renin antibody (1:100; gift from Dr. Tadashi Inagami [Vanderbilt University, Nashville, TN]), or AGT antibody (1:500; Swant). The sections were costained for anti-sarcomeric actin and laminin, where indicated. After washings, the sections were incubated with respective secondary antibodies. Specificity of the staining was determined by preadsorption 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, Temecula, CA) per the manufacturer's instructions. Cytoplasm and nuclei from the myocytes were counterstained using anti-sarcomeric actin antibody and DAPI, respectively. For cleaved caspase-3 staining, deparaffinized sections were subjected to antigen retrieval in 0.01 mol/l 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 was counted from 20 fields per heart (~25,000 cells) and three hearts per treatment group.

Reactive oxygen species detection in the heart. Superoxide production in the hearts was detected by dihydroethidium (DHE) staining (Sigma-Aldrich). Frozen heart sections (20 μm thick) were incubated with 10 μmol/l 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 15 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 are presented as an average grade.

Statistical analysis. Values are expressed as 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 not by any of the RAS inhibitors (Table 1). However, no significant effect on the heart-to-body weight ratio (Table 1) or plasma Ang II levels (Fig. 1*B*) 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. 1*A*, cardiac myocytes from diabetic rat hearts demonstrated a 9.4-fold elevation in the levels of iAng II (183 ± 13 fmol/mg protein) compared with cells from control animals (19 ± 4 fmol/mg protein). Normalization of blood glucose levels by insulin in rats administered STZ 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 that the major source of iAng II was intracellular synthesis, which is consistent with our previous report in neonatal rat ventricular myocytes (NRVMs) (14).

Intracellular synthesis of Ang II is not blocked by an ACE inhibitor. We and others had previously reported that several cell types (NRVMs, VSMCs, and renal mesan-

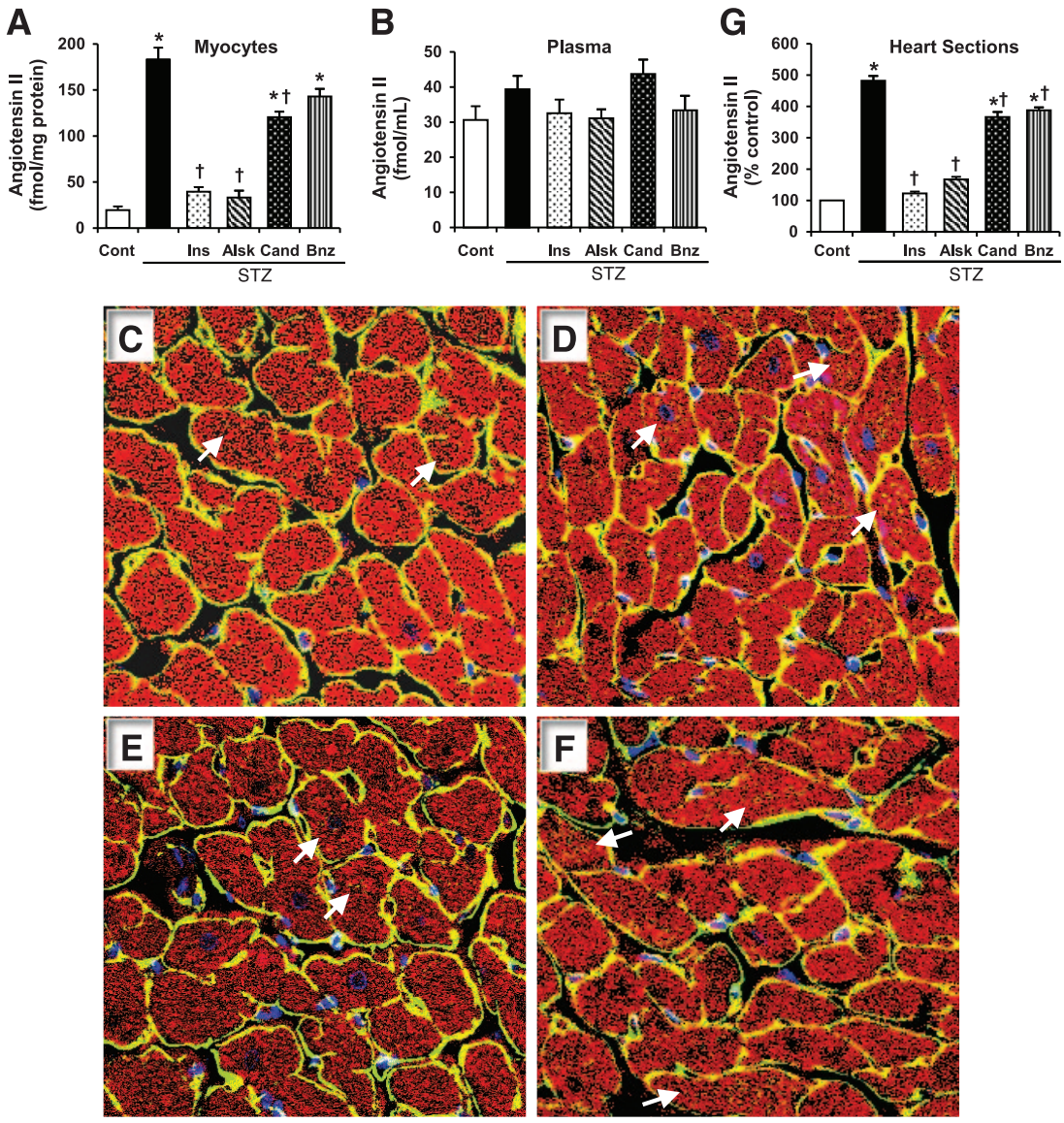

FIG. 1. Ang II levels in cardiac myocytes and plasma. Ang II was measured by a competitive ELISA in cardiac myocytes (*A*) and plasma (*B*) of control rats (Cont); diabetic rats (STZ); and diabetic rats treated with insulin (Ins), aliskiren (Alsk), candesartan (Cand), or benazepril (Bnz). Values are expressed as means ± 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 rats (*C*), diabetic rats (*D*), diabetic rats treated with aliskiren (*E*), and diabetic rats treated with candesartan (*F*). Myocyte profiles were identified by costaining with anti-sarcomeric actin (red) and laminin (yellow, peripheral staining). The blue color indicates nuclear staining by DAPI. Magnification ×1,200. *G*: Quantitative representation of Ang II fluorescence intensity in heart sections (from five images per heart and three hearts per group). Values are expressed as means ± SE, $n = 15$. *$P < 0.05$ vs. control, †$P < 0.05$ vs. diabetic rats without any treatment. (Please see http://dx.doi.org/10.2337/db08-0805 for a high-quality digital representation of this figure.)

gial cells) use 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. 1*A*, aliskiren completely normalized (33 ± 7 fmol/mg protein) iAng II levels in diabetic rat cardiac myocytes, whereas 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- to 6-h period for reactive changes in plasma levels of Ang II, which returned to baseline between 14 and 30 h after drug intake (26–28). The lack of increase in plasma Ang II levels in any of the treatment groups further indicated that the increase in cardiac myocyte Ang II levels was due to local synthesis.

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), anti-sarcomeric actin to identify cardiac myocytes (red), and DAPI to identify nuclei (blue). Significantly increased levels of Ang II staining, which colocalized with anti-sarcomeric actin, were observed in diabetic rat hearts (Fig. 1*C–F*). Quantification of fluorescence intensity revealed about a fivefold

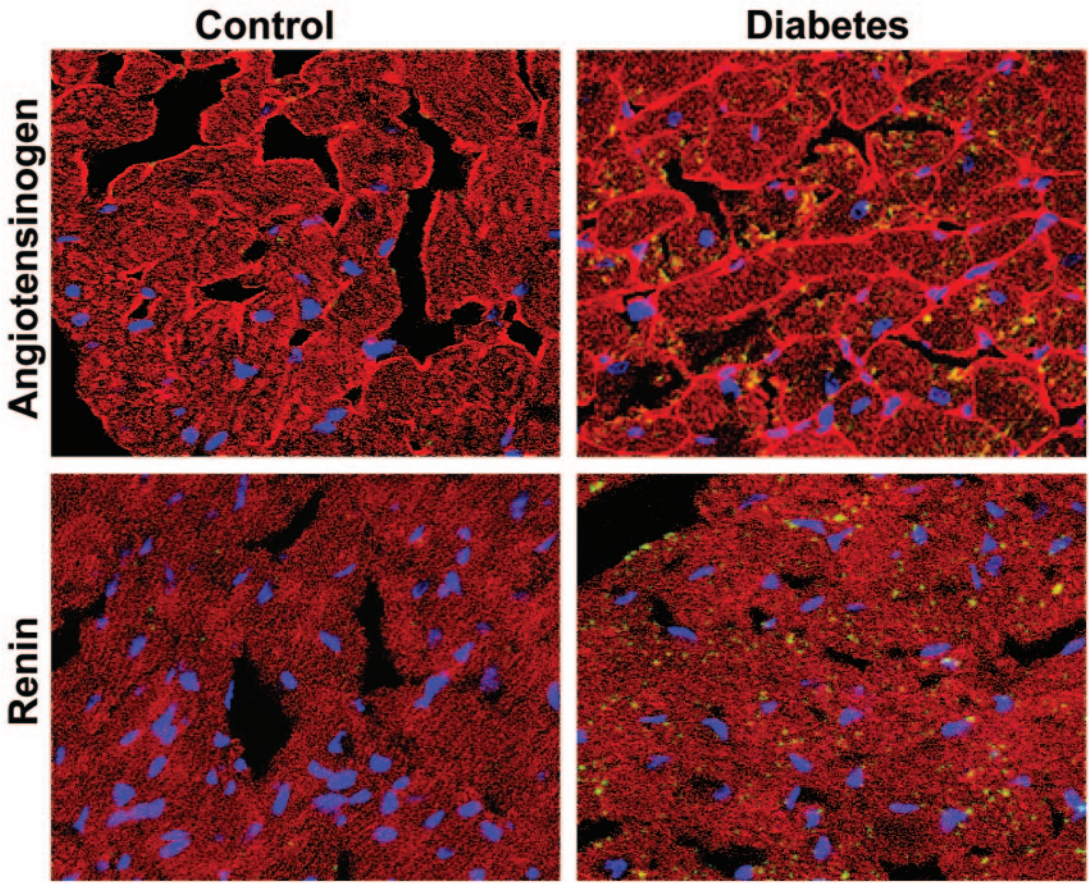

FIG. 2. Representative confocal immunofluorescence images of AGT and renin staining in hearts from control and diabetic rats. Pictures shown are merged images of staining for anti-sarcomeric actin (red), laminin (peripheral red staining in *top*), AGT (*top*) or renin (*bottom*) (green and yellow staining), and nuclei (blue). Magnification ×900. (Please see http://dx.doi.org/10.2337/db08-0805 for a high-quality digital representation of this figure.)

increase in iAng II levels (Fig. 1*G*), consistent with the Ang II measurement by ELISA (Fig. 1*A*). Immunohistochemistry also confirmed that candesartan partially reduced iAng II levels, benazepril did not have any effect, and aliskiren completely prevented the increase in iAng II in diabetic hearts (Fig. 1*G*).

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 counterstaining for anti-sarcomeric actin, as described previously (24). Elevated levels of AGT and renin were apparent in cardiac myocytes of diabetic hearts (green/yellow staining) compared with control, confirming activation of the intracellular RAS in hyperglycemic conditions (Fig. 2).

iAng 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 whether 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, whereas 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.

iAng II is correlated with hyperglycemia-induced cardiac myocyte apoptosis. Cardiac myocyte apoptosis was determined by TUNEL assay and activated caspase-3 immunostaining. Figure 4 shows an increased number of apoptotic cells in the heart sections. Quantification of apoptotic cells (Fig. 4*C* and *F*) showed a five- to eightfold increase in diabetic hearts compared with 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.

iAng 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 was evaluated on a scale of 0–4, as described in RESEARCH DESIGN

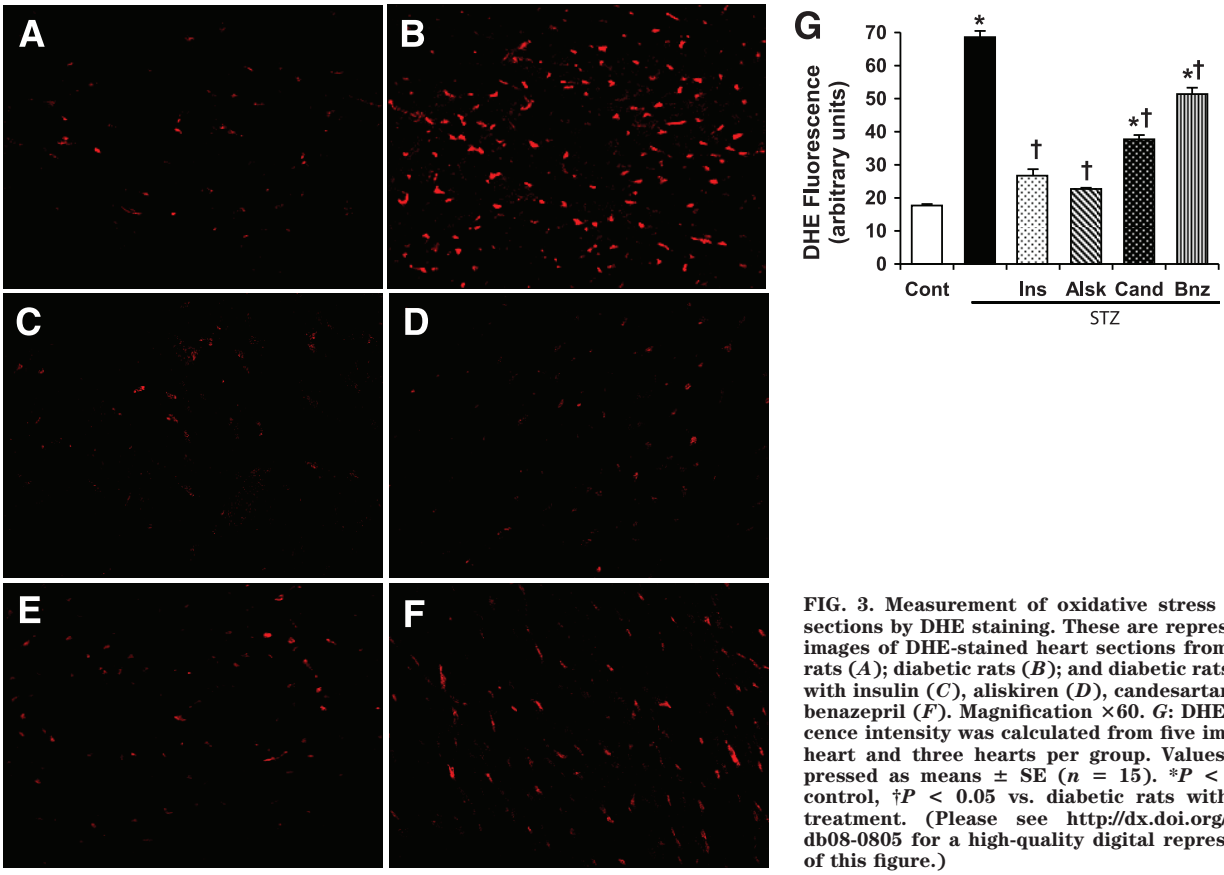

FIG. 3. Measurement of oxidative stress in heart sections by DHE staining. These are representative images of DHE-stained heart sections from control rats (*A*); diabetic rats (*B*); and diabetic rats treated with insulin (*C*), aliskiren (*D*), candesartan (*E*), or benazepril (*F*). Magnification ×60. *G*: DHE fluorescence intensity was calculated from five images per heart and three hearts per group. Values are expressed as means ± SE (n = 15). *P < 0.05 vs. control, †P < 0.05 vs. diabetic rats without any treatment. (Please see http://dx.doi.org/10.2337/db08-0805 for a high-quality digital representation of this figure.)

AND METHODS. Even after only 1 week of diabetes, the overall staining for fibrosis was enhanced in hearts from diabetic rats (grade 1.5) compared with 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, respec-

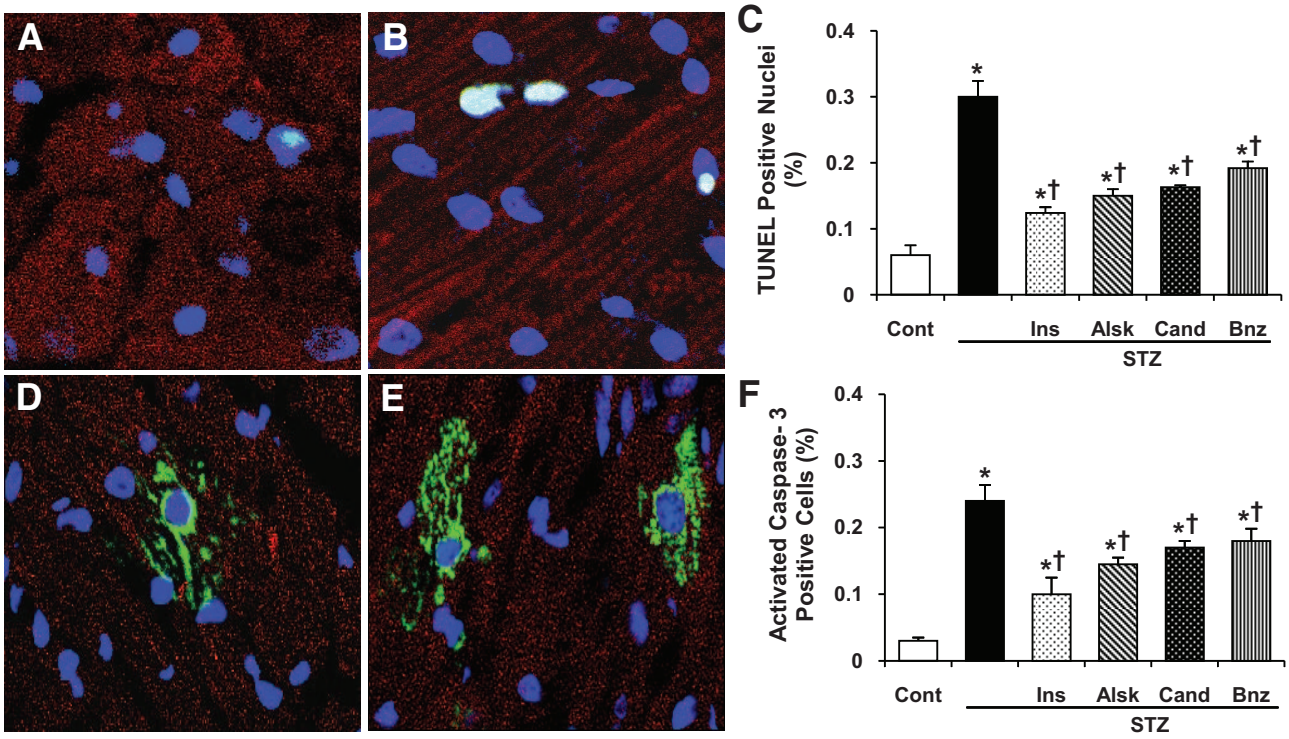

FIG. 4. Detection of apoptosis in cardiac myocytes by TUNEL assay and cleaved caspase-3 staining. *A* and *B*: TUNEL assay on heart sections from control (*A*) and diabetic (*B*) rats. *D* and *E*: Staining for cleaved caspase-3 in the hearts of control (*D*) and diabetic (*E*) rats. *C* and *F*: Quantification of TUNEL$^+$ (*C*) and cleaved caspase-3$^+$ (*F*) cells (~25,000 cells were counted in each case). Values are expressed as means ± SE. *P < 0.05 vs. control, †P < 0.05 vs. diabetic rats without any treatment. (Please see http://dx.doi.org/10.2337/db08-0805 for a high-quality digital representation of this figure.)

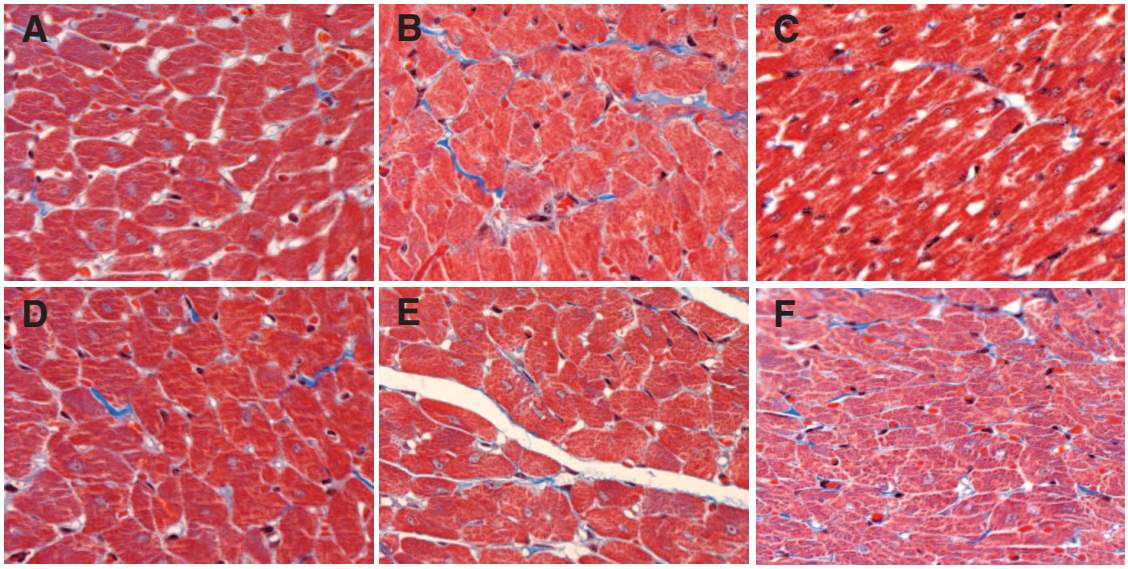

FIG. 5. Detection of cardiac fibrosis by Masson's Trichrome staining in heart sections from control rats (*A*); diabetic rats (*B*); and diabetic rats treated with insulin (*C*), aliskiren (*D*), candesartan (*E*), or benazepril (*F*). Representative images of five sections per heart and three hearts per group were observed with a ×40 objective. (Please see http://dx.doi.org/10.2337/db08-0805 for a high-quality digital representation of this figure.)

tively), 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 with inhibition with an AT_1 antagonist or ACE inhibitor.

We first described a physiologically relevant intracellular RAS in NRVMs (14), defined as the presence of the precursor protein and enzymes and 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 NRVMs, the intracellular accumulation of RAS components and iAng II synthesis have also been described in VSMCs 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 threefold increase in Ang II staining was described in hearts from diabetic compared with nondiabetic patients, which was enhanced an additional 2.5-fold in diabetic hypertensive patients (30). However, in that study, it was not clear whether 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 AT_1 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, because 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 AT_1 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 NRVMs after exposure to high glucose, whereas 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 after prolonged treatment with ACE inhibitors (34). In the present study, the lack of reduction in iAng II levels after only 1 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 VSMCs 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 severalfold in diabetes, which

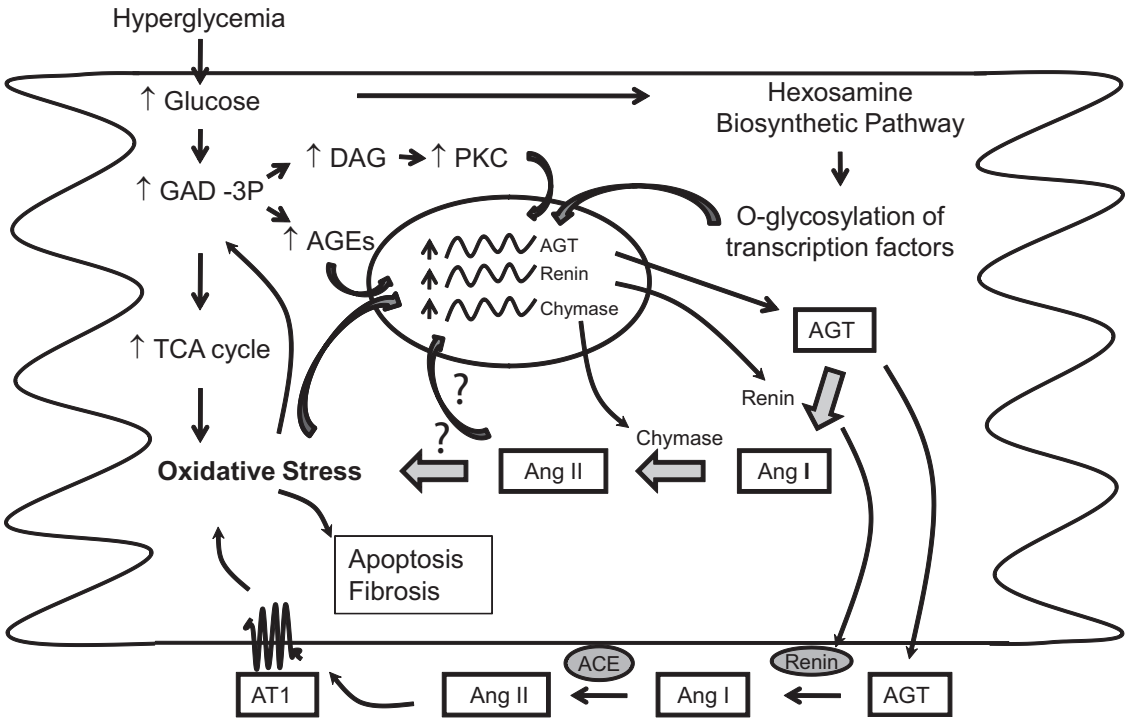

FIG. 6. Schematic representation of the relationship between hyperglycemia, iAng II, and pathological effects. In hyperglycemia, there is an increase in glucose oxidation through the tricarboxylic acid cycle in mitochondria, which results in enhanced generation of reactive oxygen species. Overproduction of superoxide inhibits glyceraldehyde-3-phosphate dehydrogenase activity, resulting in an accumulation of upstream metabolites of the glycolytic pathway. Increased levels of glyceraldehyde-3-phosphate (GAD-3P) cause 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 of these products of hyperglycemia, i.e., oxidative stress, AGEs, PKC, and *o*-glycosylation of transcription factors, activate expression of RAS components. Cardiac myocytes synthesize and retain Ang II intracellularly in hyperglycemia, whereas 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 AT_1 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; whereas 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.

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 preprofragment, eliminating the need for proteolytic activation (43). We have previously described a significant increase in intracellular renin levels in NRVMs 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.

Although aliskiren is the most potent inhibitor of human renin (half-maximal inhibitory concentration [IC_{50}] 0.6 nmol/l), it inhibits rat renin at higher concentrations (IC_{50} 80 nmol/l) (44). We chose an aliskiren dose of 30 $mg \cdot kg^{-1} \cdot day^{-1}$ 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 study) demonstrated that an oral dose of 30 mg/kg resulted in an area under the curve of 3.06 ± 1.8 $\mu mol \cdot l^{-1} \cdot h^{-1}$, 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 on 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 after 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 $mg \cdot kg^{-1} \cdot day^{-1}$ for 2 weeks, showed a kidney-to-plasma ratio of aliskiren in the range of 45 to 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 suggest slow clearance and accumulation in tissues (48). The 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. Furthermore, tissue accumulation of aliskiren may result in effects on target organs, even at doses that 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 AT_1-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 AT_1 receptor, demonstrated that some of the effects of iAng II do not require AT_1 receptor (22). In addition, enhanced transforming growth factor-β/smad signaling was reported in kidneys from diabetic AT_1-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 with candesartan or benazepril. Effects of aliskiren were unlikely to be mediated through Ang II–independent mechanisms because aliskiren does not inhibit renin binding to the (pro)renin receptor and extracellular signal–related kinase 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. Although STZ-induced diabetes is representative of type 1 diabetes, we 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 NRVMs 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 protein kinase C (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 (52), 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; whereas 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 NRVMs, 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 (53). 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 (10,54–56). Although 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 peroxisome proliferator–activated receptor-γ and the kallikrein-kinin system (57–59); 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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