Original Article

Contributions of Inflammation and Cardiac Matrix Metalloproteinase Activity to Cardiac Failure in Diabetic Cardiomyopathy

The Role of Angiotensin Type 1 Receptor Antagonism

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We investigated the effect of the angiotensin type 1 (AT-1) receptor antagonist, irbesartan, on matrix metalloproteinase (MMP) activity and cardiac cytokines in an animal model of diabetic cardiomyopathy. Diabetes was induced in 20 C57/bl6 mice by injection of streptozotocin (STZ). These animals were treated with irbesartan or placebo and were compared with nondiabetic controls. Left ventricular (LV) function was measured by pressure-volume loops with parameters for systolic function (end systolic elastance [Ees]) and diastolic function (cardiac stiffness) 8 weeks after STZ treatment. The cardiac protein content of interleukin (IL)1β and transforming growth factor (TGF)β1 were measured by enzyme-linked immunosorbent assay. The total cardiac collagen content and collagen type 1 and 3 were measured by histochemestry, and MMP-2 activity was measured by gelatin zymography. LV dysfunction was documented by impaired Ees and diastolic stiffness in STZ mice compared with controls. This was accompanied by increased TGFβ, IL1β, and fibrosis and decreased MMP-2 activity. Treatment with irbesartan attenuated LV dysfunction, IL1β, TGFβ, and cardiac fibrosis compared with untreated diabetic animals and normalized MMP activity. These findings present evidence that AT-1 receptor antagonists attenuate cardiac failure by decreasing cardiac inflammation and normalizing MMP activity, leading to normalized cardiac fibrosis in STZ-induced diabetic cardiomyopathy. *Diabetes* 56:641–646, 2007

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ANGII, angiotensin II; ARB, angiotensin receptor blocker; AT-1, angiotensin type 1; dP/dt max, contractility; dP/dt min, relaxation; Ees, end systolic elastance; IL, interleukin; LV, left ventricular; LVEDP, LV end diastolic pressure; MMP, matrix metalloproteinase; STZ, streptozotocin; TGF, transforming growth factor.

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Diabetic cardiomyopathy is one of the leading causes of increased morbidity and mortality in the diabetic population. It is defined as left ventricular (LV) dysfunction independent of atherosclerosis, coronary heart disease, or hypertension (1,2). Diverse pathogenic mechanisms contribute to diabetic cardiomyopathy, including changes in the composition of the extracellular matrix with enhanced cardiac fibrosis and increased cardiac cytokine levels (3,4). These pathological mechanisms are mediated by hyperglycaemia (5) and by increased aldosterone and angiotensin II (ANGII) levels (6). Cardiac cytokines that are stimulated by ANGII, such as interleukin (IL)1β and transforming growth factor (TGF)β1, are involved in the development of cardiac fibrosis and cardiac failure (7–10). Furthermore, next to nonenzymatic glycosylation of collagens, a dysregulation of collagen degrading matrix metalloproteinases (MMPs) and their tissue inhibitors is supposed to be a hallmark for myocardial fibrosis in diabetes. Thus far, there is little information regarding MMPs and and their tissue inhibitors in diabetic cardiomyopathy.

Angiotensin type 1 (AT-1) receptor antagonists (angiotensin receptor blockers [ARBs]) are, next to ACE inhibitors, widely used in clinical practice in congestive heart failure. They show multiple beneficial effects, such as preventing LV remodeling after myocardial infarction, reducing hypertrophy in hypertensive heart disease, and improving systolic and diastolic function. Therefore, we hypothesized that ARBs play a role in cardiac extracellular matrix regulation under diabetic conditions.

To examine their role in diabetic cardiomyopathy, we investigated the systolic and diastolic LV function in an animal model of streptozotocin (STZ)-induced diabetes. Further, we analyzed changes in the cardiac extracellular matrix and cytokine levels in diabetic cardiomyopathy after chronic treatment with the ARB irbesartan.

RESEARCH DESIGN AND METHODS

Diabetes was induced in 20 C57i/BL6J mice by injection of STZ (50 mg/kg i.p. for 5 days) dissolved in 0.1 mol/l sodium citrate, pH 4.5, as previously described (11). This dose is known to induce no acute renal failure in C57i/BL6J mice (12). Hyperglycemia (glucose >22 mmol/l) was confirmed 7 days later using a reflectance meter (Acutrend; Boehringer, Mannheim,

TABLE 1
Animal characterization

	Control	STZ	STZ-IRB
Blood pressure			
SAP at the beginning of the study (mmHg)	114.5 ± 5.8	119.1 ± 4.1	121.6 ± 4.2
SAP at the end of the study (mmHg)	117 ± 6.9	69 ± 1.3*	72 ± 1.9*
Body and heart weight			
Body weight at the beginning of the study (g)	21.2 ± 1.2	20.4 ± 1.3	22.1 ± 1.4
Body weight at the end of study (g)	28.2 ± 1.2	18.1 ± 1.1*	17.6 ± 1.2*
Heart weight (mg)	132 ± 5	89 ± 3*	90 ± 4*
Heart/body weight (mg/g)	4.7 ± 0.4	4.9 ± 0.6	5.0 ± 0.4
Glucose levels			
Glucose level after 8 weeks of diabetes (mmol/l)	4.4 ± 0.3	34.6 ± 1.9*	36.4 ± 1.3

General characteristics of controls, mice with STZ-induced diabetes, and diabetic mice treated with irbesartan (STZ-IRB). *$P < 0.05$ vs. controls. SAP, systolic arterial pressure.

Germany), as well as at the end of the study (glucose >30 mmol/l). Vehicle-treated mice served as nondiabetic controls ($n = 10$). Diabetic mice were treated with the ARB irbersatan (STZ-IRB, $n = 10$, 20 mg/kg body wt daily p.o.) or placebo (STZ, $n = 10$) for 8 weeks. The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH publ. no. 85-23, revised 1985).

Surgical procedures and hemodynamic measurements. Systolic arterial pressure (mmHg) was analyzed before injection of STZ and at the end of the study before final LV catherization. The animals were anesthetized (125 mg/g thiopental i.p.), intubated, and artificially ventilated. As recently described (13), a 1.4-F microconductance pressure catheter (ARIA SPR-719; Millar-Instruments, Houston, TX) was positioned in the LV for registration of LV pressure–volume loops in a closed-chest model. Indexes of cardiac function were derived from pressure-volume data obtained both at steady state and during transient preload reduction by occlusion. Systolic function was quantified by LV end systolic pressure (mmHg), contractility (dP/dt max; mmHg/s), and by the slope of the end systolic pressure–volume relationship (end systolic elastance [Ees]) as an index of LV contractility and by ejection fraction. Global cardiac function was quantified by the end systolic volume (μl), end diastolic volume (μl), stroke volume (μl), cardiac output (μl/min), and heart rate (beat/min). Diastolic function was measured by LV end diastolic pressure (LVEDP, mmHg), relaxation (dP/dt min; mmHg/s), and the end diastolic pressure–volume relationship (stiffness), an indicator for LV cardiac stiffness.

Zymography of MMP-2 activity. Gelatin zymography was performed to determine gelatinolytic activities of MMP-2. Myocardial protein (40 μg) was treated with sampling buffer (0.5 mol/l Tris-HCl, glycerol, 10% SDS, and 0.1% bromphenol blue) in a final solution of 20 μl. SDS-PAGE was performed using 10% polyacrylamid gel containing 0.1% gelatin at 125 V for 60 min. SDS was removed with Triton X-100 for 60 min, and the gel was incubated in a developing buffer (Tris-base, Tris-HCl, NaCl, $CaCl_2$, Brij-35, and $ZnCl_2$) overnight. Gels were stained for 3 h with 0.5% coomassie G250 and destained for 60 min in 7% acetic acid and 35% methanol. The gelatinolytic activities were detected as a clear band against a blue background (arbitrary unit per centimeter) and analyzed as relative optical densities using Scion Image software.

Cardiac IL1β and TGFβ protein content. IL1β and TGFβ protein content was determined by enzyme-linked immunosorbent assay from Pharmingen/BD Biosciences (San Diego, CA). All assays were done twice, and the absorbance values were equalized with the absorbance values of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (Active Motif, Carlsbad, CA).

Histological assessment of MMP-2, MMP-9, collagen type 1, collagen type 3, and total collagen. Immunohistochemistry was carried out using primary antibodies (MMP-2, MMP-9, collagen type 1, and collagen type 3; Chemicon, Temecula, CA) followed by the DAKO Envision HRP technique (DAKO, Glostrup, Denmark). Total collagen content was stained using the Sirius Red technique (Polyscience, Warrington, PA). All stained sections were quantified by digital image analysis (11).

Statistical analysis. All data are expressed as means ± SE. Statistical significance between multiple groups was determined using ANOVA and post hoc analysis with a Bonferroni test. Values of $P < 0.05$ were considered significant.

RESULTS

Body weight decreased in both STZ and STZ-IRB mice after 8 weeks of STZ-induced diabetes. Glucose levels were highly increased but did not differ between both diabetic groups. Further data are presented in Table 1.

Cardiac performance. Heart rate was significantly decreased in STZ mice compared with controls (−30%, $P <$

TABLE 2
Hemodynamic parameters

	Control	STZ	STZ-IRB
Systolic function			
LVP (mmHg)	107.5 ± 6.8	67.1 ± 3.1*	69.6 ± 2.2*
dP/dt max (mmHg/s)	6,021 ± 609	3,556 ± 323*	4,085 ± 379*
Ejection fraction (%)	61.7 ± 1.9	48 ± 5	55 ± 4
Ees (mmHg/μl)	1.67 ± 0.08	0.95 ± 0.07*	1.45 ± 0.03§
Diastolic function			
LVEDP (mmHg)	3.5 ± 0.6	11.8 ± 2.6*	7.3 ± 1.2*§
dP/dt min (mmHg/s)	−5,402 ± 547	−3,270 ± 323*	−3,764 ± 411*
Diastolic stiffness (ml^{-1})	0.038 ± 0.003	0.109 ± 0.009*	0.059 ± 0.004*§
Cardiac performance			
Heart rate (bpm)	434 ± 23	311 ± 35*	371 ± 12
Cardiac output (μl/min)	16,053 ± 635	8,310 ± 825*	11,590 ± 1,071*§
Stroke volume (μl)	36.9 ± 1.2	27.7 ± 2.6	31.9 ± 5.2
Volume end diastolic (μl)	63.9 ± 4	64 ± 3	69.7 ± 5
Volume end systolic (μl)	23.1 ± 8	36 ± 8	37 ± 8
Afterload (mmHg/μl)	2.9 ± 0.08	2.7 ± 0.05	2.3 ± 0.04§

Hemodynamic function of controls, mice with STZ-induced diabetes, and diabetic mice treated with irbesartan (STZ-IRB). *$P < 0.05$ vs. controls; §$P < 0.05$ vs. STZ.

TABLE 3
Cardiac collagen content

	Control	STZ	STZ-IRB
Total collagen content Sirius Red (% AF)	2.6 ± 0.4	7.5 ± 1.1	3.6 ± 0.8
Collagen type I content (% AF)	0.53 ± 0.09	1.03 ± 0.21*	0.28 ± 0.03
Collagen type III content (% AF)	7.6 ± 0.9	17.5 ± 2.3*	7.73 ± 1.1

Cardiac collagen content of controls, mice with STZ-induced diabetes, and diabetic mice treated with irbesartan (STZ-IRB), showing increased total collagen and collagen type 1 and type 3 content in STZ mice, normalized in STZ-IRB. *$P < 0.05$ vs. controls and STZ. AF, area fraction.

0.05) due to the known effect of diabetic cardiac autonomy (14); however, due to treatment with irbesartan, it was normalized. The afterload was decreased, due to treatment with irbesartan, when compared with either control (−21%, $P < 0.05$) or STZ (−16%, $P < 0.05$) mice. The cardiac output was significantly decreased when STZ was compared with controls (−50%, $P < 0.05$). This decline of cardiac output could be partly prevented by irbesartan (+39%, $P < 0.05$) but was still impaired when STZ-IRB was compared with controls (−28%, $P < 0.05$) (Table 2 and Fig. 1).

Systolic function. All load-dependent systolic parameters were significantly decreased when STZ was compared with the control group: end systolic pressure (−38%, $P < 0.05$), dP/dt max (−41%, $P < 0.05$), and ejection fraction (−23%, $P < 0.05$). The load-independent parameter Ees was significantly reduced (−43%, $P < 0.05$). Treatment with irbesartan led to an improvement of Ees when STZ-IRB was compared with STZ mice (+52%, $P < 0.05$) but was still decreased when STZ-IRB was compared with controls (−14%, $P < 0.05$) (Table 2 and Fig. 1).

Diastolic function. The diastolic parameters were significantly aggravated when STZ was compared with the control group: dP/dt min (+40%, $P < 0.05$), LVEDP (+330%, $P < 0.05$), diastolic cardiac stiffness (+290%, $P < 0.05$). Irbesartan improved diastolic function with a reduction of LVEDP (−39%, $P < 0.05$) and diastolic cardiac stiffness (−46%, $P < 0.05$) when STZ-IRB was compared with STZ. Nevertheless, LVEDP (+200%, $P < 0.05$) and diastolic cardiac stiffness (+155%, $P < 0.05$) were still significantly impaired when STZ-IRB was compared with controls (Table 2 and Fig. 1).

Myocardial IL1β and TGFβ levels. In the myocardium of the diabetic mice, the cardiac protein level of IL1β (+110%, $P < 0.05$) was significantly increased in STZ compared with control mice. This upregulation was prevented by irbesartan, with no significant difference when STZ-IRB was compared with controls. The protein content of TGFβ (+30%, $P < 0.05$) was significantly increased in the STZ group compared with controls. This could be normalized by irbesartan (Fig. 2).

MMP-2 gelatinolytic activity. The gelatinolitic activity of MMP-2 was significantly decreased in the STZ group (−30%, $P < 0.05$) compared with the control group. This was normalized by irbesartan in the STZ-IRB group (Fig. 3).

Myocardial protein levels of MMP-2 and -9. MMP-2 protein content was significantly downregulated in STZ mice (−60%, $P < 0.05$) but not significantly different in the STZ-IRB group compared with controls. Differently, MMP-9 protein content was significantly upregulated in STZ mice (+50%, $P < 0.05$) and normalized in STZ-IRB compared with controls. (Fig. 4).

Cardiac collagen content. Total collagen content was increased in STZ mice (+220%, $P < 0.05$) compared with controls. Irbesartan treatment normalized total collagen content in STZ-IRB compared with controls. Both, collagen type 1 and type 3 were increased in STZ (+100 and +160%, respectively, $P < 0.05$) compared with controls. Again, treatment with irbesartan normalized collagen type 1 and type 3 content to control levels (Fig. 5 and Table 3).

DISCUSSION

The key finding of the current study is that the ARB irbesartan improved the dysregulation of the cardiac extracellular matrix in the development of STZ-induced diabetic cardiomyopathy. Irbesartan treatment normalized MMP-2 activity, reduced cardiac TGFβ and IL1β levels, and decreased cardiac fibrosis. These changes were associated with improved LV function, despite severe hyperglycemic conditions.

LV function. Cardiac fibrosis is one of the main modulators of diastolic cardiac stiffness (15). Consequently, increased collagen content in the STZ group led to diastolic dysfunction. This is evident from the increased diastolic cardiac stiffness, a load-independent parameter for dia-

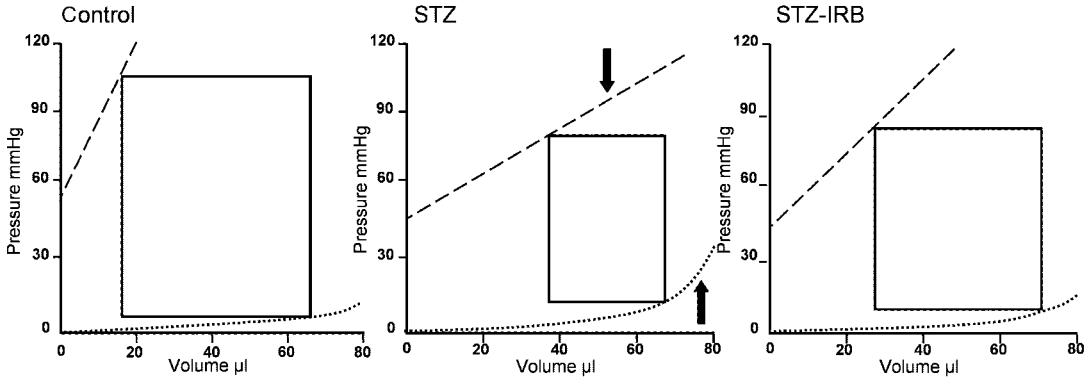

FIG. 1. Schematic pressure volume loops of controls, mice with STZ-induced diabetes, and diabetic mice treated with irbesartan (STZ-IRB), showing impaired LV function in STZ (black arrows indicating decreased Ees for systolic dysfunction and increased stiffness for diastolic dysfunction) and improved LV function due to irbesartan treatment. Furthermore, the area of the pressure-volume loop is decreased in STZ mice and improved due to treatment with irbesartan as sign of improved cardiac function.

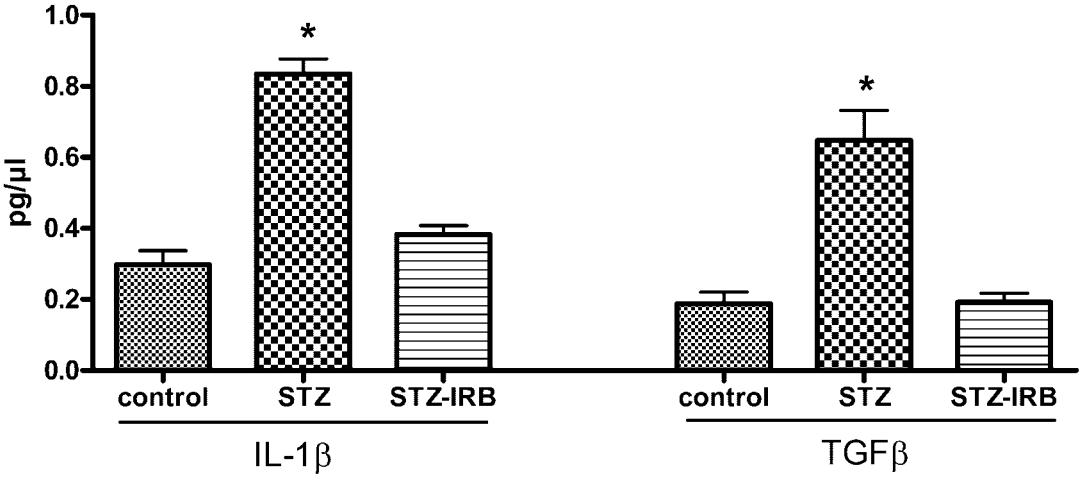

FIG. 2. Cardiac content of TGFβ and IL1β measured by enzyme-linked immunosorbent assay of controls, mice with STZ-induced diabetes, and diabetic mice treated with irbesartan (STZ-IRB), showing increased levels of TGFβ and IL1β in STZ mice, both normalized in STZ-IRB. *$P < 0.05$ vs. control and STZ-IRB mice.

stolic function, as well as from the increased LVEDP, a marker for actual load in diastole, in the STZ group. Treatment with irbesartan improved diastolic function and decreased both the diastolic cardiac stiffness and the LVEDP.

Furthermore, the load-dependent parameter for systolic function—end systolic pressure and contractility—were significantly decreased in the STZ group, which is in agreement with the well-established model of STZ-induced diabetic cardiomyopathy (16–19). Interestingly, irbesartan did not influence the end systolic pressure when STZ and STZ-IRB were compared. This can be explained by the decreased afterload in the STZ-IRB group, which is well known as a vasodilatory hemodynamic effect of ARBs. Nevertheless, the Ees (the load-independent parameter for systolic function) was increased by irbesartan, and this was accompanied by increased cardiac output. These are clear indicators of improved systolic function.

Cardiac cytokine and growth factor levels. Recently, we showed that diabetic cardiomyopathy is accompanied by increased cardiac cytokine levels (4). Consistently, we found increased IL1β and TGFβ levels under diabetic conditions in the STZ group in the present study. Cytokines can attenuate myocyte contractility directly through alterations in sarcoplasmic reticulum function and indirectly by downregulating the sarcoplasmic calcium ATPase expression (20). Moreover, they are also known to regulate the extracellular matrix. TGFβ acts as a profibrotic growth factor by upregulating the connective tissue growth factor through gene transcription (21). TGFβ itself is upregulated by ANGII via the AT-1 receptor, as shown in obstructive nephropathy (22), or by chronic ANGII infusion (23). On the other hand, IL1β induces MMP expression as a potential counterregulator of the profibrotic TGFβ (24). The blockade of the AT-1 receptor normalized the protein content of TGFβ and IL1β in our study. Several mechanisms are presumed to mediate the anti-inflammatory effects of ARBs, including the decrease of invading macrophages resulting from downregulation of the chemokine monocyte chemoattractant protein-1 (25), suppression of T-lymphocytes activation (26), and direct gene regulation by nuclear factor-κB (27). Furthermore, it has been shown that inflammatory cytokines regulate tissue inhibitors of metalloproteinases, which in turn regulate the inflammatory cytokines (28,29).

Cardiac fibrosis. Accumulation of cardiac fibrosis is a general hallmark of heart failure. Excessive tissue fibrosis is regulated by, for example, high glucose levels, ANGII, aldosterone, and TGFβ, all upregulated under diabetic conditions (30,31). Accumulation of cardiac fibrosis can result on the one hand from excessive production of collagen by fibroblasts and on the other hand from decreased degradation of collagen by MMPs. The effect of these mediators on the extracellular matrix and the effects of ARBs in diabetic cardiomyopathy are still uncertain.

Extracellular matrix regulation. Increased total collagen content in human diabetic cardiomyopathy, as well as in our model, is accompanied with maladaptive changes in the composition of the extracellular cardiac matrix. MMPs are critically involved in this turnover by degrading collagens in cardiac tissue (32). Numerous MMPs are known, but we focused on MMP-2 and -9 (both gelatinases) because of their effect on cardiac extracellular matrix. Little is known about cardiac MMP levels in diabetes,

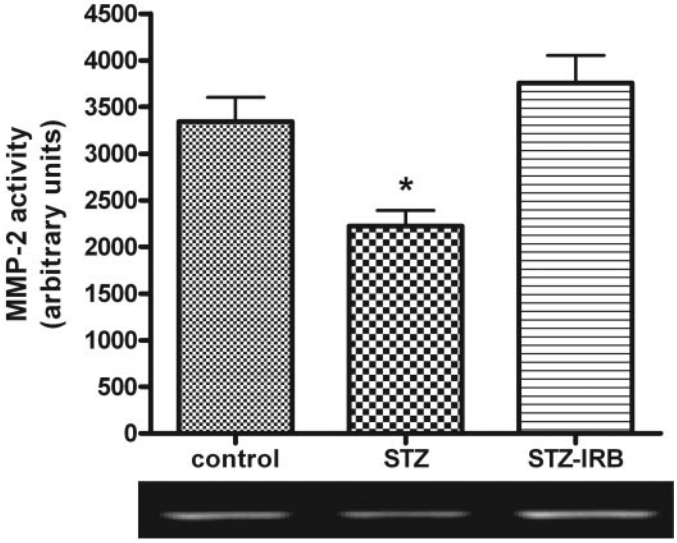

FIG. 3. MMP-2 activity showing decreased proteolitic activity in mice with STZ-induced diabetes, normalized in diabetic mice treated with ibesartan (STZ-IRB) compared with nondiabetic controls by zymography. *$P < 0.05$ vs. control and STZ-IRB

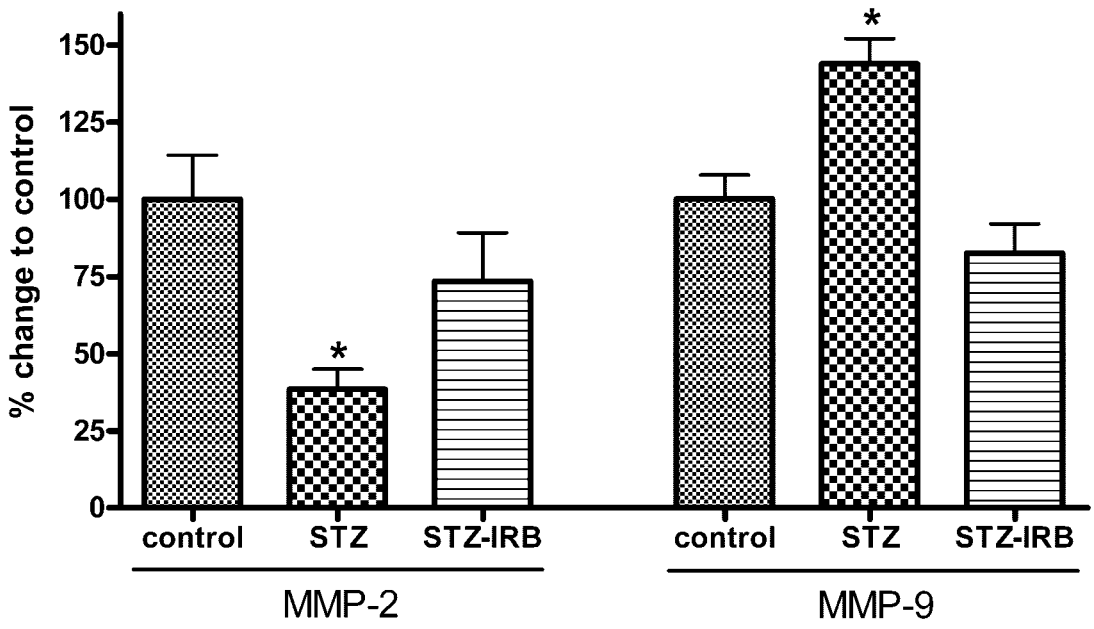

FIG. 4. MMP-2 and -9 protein content measured by immunohistochemestry in controls, mice with STZ-induced diabetes, and diabetic mice treated with irbesartan (STZ-IRB), showing downregulated MMP-2 and upregulated MMP-9 levels in STZ mice, normalized in STZ-IRB. *$P < 0.05$ vs. controls.

especially as a similar regulation of MMPs has not been demonstrated in different tissues (33).

Here, we demonstrate significantly decreased protein content of MMP-2 in STZ-induced diabetes. MMP-2 mainly degrades fibrillar collagen peptides and newly synthesized collagen fibers. Consistent with increased cardiac fibrosis and decreased MMP-2 content, zymography revealed decreased MMP-2 activity in the cardiac tissue of diabetic animals. MMP-9 was upregulated in STZ-induced diabetic cardiomyopathy in our study. MMP-9 has the same structural protein substrates as MMP-2, but its proteolytic activity is much lower, and, therefore, it might not be significant for altering collagen content. Notably, MMP-9, but not other MMPs, upregulate biologically active proteins such as the profibrotic growth facotor TGFβ (24). This may represent a feedback mechanism and may contribute to increased collagen accumulation. Our findings agree with studies on cultured cardiac fibroblasts that have demonstrated that the MMP-2 activity was downregulated by high glucose levels and by ANGII (34). It is known that low proteolytic activity of MMPs contributes to diabetes-induced renal fibrosis (35). We therefore conclude that a decrease in cardiac MMP-2 activity translates into less collagen degradation and thus promotes cardiac fibrosis. Treatment with irbesartan is not only associated with normalized activity of MMP-2 in our study, but also with

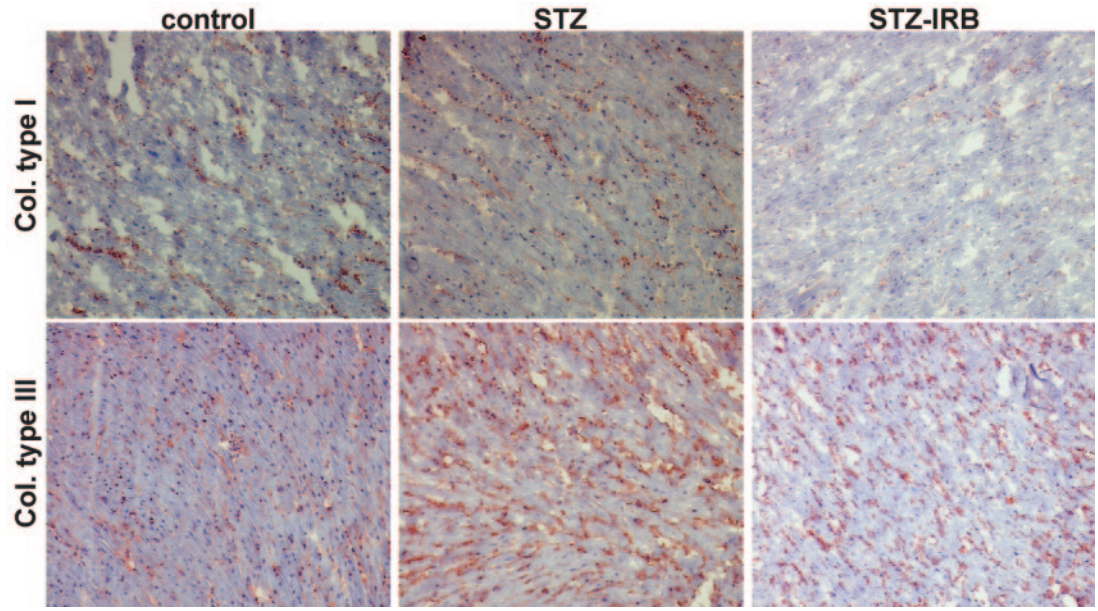

FIG. 5. Representative immunohistological pictures of cardiac collagen in controls, mice with STZ-induced diabetes, and diabetic mice treated with irbesartan (STZ-IRB), showing increased cardiac collagen type 1 (Col. type 1) and collagen type 2 (Col. type 2) in STZ mice, normalized in STZ-IRB.

normalized MMP-2 and -9 protein levels. These findings indicate that the AT-1 receptor is involved in regulating cardiac MMP protein content and MMP-2 activity in diabetes, possibly by reducing IL1β and TGFβ levels in the cardiac tissue, as hyperglycemia was not affected.

It is known that diabetic cardiomyopathy is accompanied by cardiac fibrosis. This excessive accumulation of collagen fibers in the interstitium of the heart was associated with increased IL1β and TGFβ levels and low proteolytic activity of MMP-2 in the current study. The present experiment showed that AT-1 receptor antagonism prevented cardiac fibrosis in STZ-induced cardiomyopathy, most likely by normalizing the MMP activity and the cardiac cytokine and growth factor content. Since it is possible that subtherapeutic insulin administration to STZ-injected mice may be the better model for human diabetic cardiomyopathy, the influence of insulin treatment on the effect of ARBs has to be evaluated.

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