Effects of eplerenone on the activation of matrix metalloproteinase-2 stimulated by high glucose and interleukin-1β in human cardiac fibroblasts

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ABSTRACT. The aim of this study was to determine the influence of high glucose (HG) and interleukin (IL)-1β on human cardiac fibroblast (HCF) functions, and to evaluate the effects of eplerenone in these responses. HCFs were cultured in normal or HG media in the absence or presence of IL-1β and/or eplerenone. We assessed matrix metalloproteinase-2 (MMP-2) activity in the supernatant by in-gel zymography, and determined mRNA expression levels of MMP-2 and tissue inhibitor of metalloproteinase-2 (TIMP-2) by reverse transcription-polymerase chain reaction. Equimolar D-mannitol was used as an osmotic control. HG stimulated MMP-2 activity and promoted MMP-2 mRNA synthesis. Increased effects were also observed in equimolar D-mannitol treatments, but these effects were weaker compared to those of glucose. The combination of HG and

IL-1β resulted in a 2-fold increase in MMP-2 activity and mRNA expression compared with HG or IL-1β alone. Increases in HG- or IL-1β-induced MMP-2 activity and mRNA expression were blocked by eplerenone. Neither HG nor IL-1β affected TIMP-2 mRNA expression. HG increased MMP-2 activity by regulation of MMP-2 mRNA expression in HCFs through osmotic and non-osmotic pathways. Synergistic effects of IL-1β added to HG media on MMP-2 activity and mRNA expression were observed in HCFs. Eplerenone normalized the effect of MMP-2 activity and HG- or IL-1β-induced expression in HCFs.

Key words: Fibroblasts; High glucose; Matrix metalloproteinase-2; Interleukin-1β; Mineralocorticoid receptor antagonists

INTRODUCTION

Diabetic cardiomyopathy is a cardiac dysfunction in diabetes without coronary atherosclerosis. Changes in ventricular structure and left ventricular systolic and diastolic dysfunction have all been observed in patients with diabetic cardiomyopathy (Bauters et al., 2003). High glucose (HG) levels can induce the development of diabetic cardiomyopathy; however, the underlying mechanism remains unknown. Several possible mechanisms have been proposed, including: 1) intramyocardial microangiopathy, 2) inflammation, and 3) alterations in the extracellular matrix (ECM) (Bauters et al., 2003; Tsioufis et al., 2012; Miki et al., 2013).

The balance of collagen synthesis and degradation plays an important role in the homeostasis of the ECM under non-pathological conditions (Spinale, 2007). Cardiac fibroblasts constitute one of the major cell types contributing to structural and functional properties of the heart. Cardiac fibroblasts produce ECM and secrete matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs). MMPs degrade ECM structural proteins (Jun et al., 2011), and TIMPs are the major endogenous regulators of MMP activities in tissues (Kandalam et al., 2011). MMPs and their tissue inhibitors represent a regulatory system that plays a crucial role in ECM metabolism (Kosmala et al., 2008). A previous study found that HG altered the level of MMPs and cytokines in fibroblasts (Westermann et al., 2007). Furthermore, increased pro-inflammatory cytokines have been found in diabetic animal models and diabetic patients (Ersoy et al., 2004; Everard et al., 2012). Moreover, interleukin-1β (IL-1β) and tumor necrosis factor-α were shown to increase the expression and activity of MMP-2 and MMP-9 in cardiac fibroblasts (Siwik et al., 2000). However, the influence of pro-inflammatory cytokines on MMP-2 activity in fibroblasts incubating with HG has not yet been well elucidated.

Mineralocorticoid receptor antagonists can decrease morbidity and mortality in patients with left ventricular systolic dysfunction and heart failure, with or without diabetes mellitus, after myocardial infarction (Pitt et al., 2003). Treatment with the mineralocorticoid receptor antagonist eplerenone has been shown to decrease MMP-2 protein secretion and mRNA expression in animal heart-failure models (Rastogi et al., 2007). However, whether eplerenone might improve the prognosis of diabetic cardiomyopathy through altering MMP-2/TIMP-2 remains unknown.

The purpose of this study was to verify the effects of HG and IL-1β on MMP-2 and TIMP-2 levels in cultured human cardiac fibroblasts (HCFs). Furthermore, we evaluated whether eplerenone could affect the activity of MMP-2 induced by HG and IL-1β in HCFs.

MATERIAL AND METHODS

Materials

The 4N0-500 and 4N3-500-S media were purchased from Cell Systems (Kirkland, WA, USA). Human recombinant IL-1β was purchased from Peprotech (Rocky Hill, NJ, USA). Eplerenone was kindly supplied by Pfizer Inc. All other chemicals were commercially available and of reagent grade.

Cell culture

Primary HCFs were purchased at passage 2 from Cell Systems. HCFs were seeded on 6-well tissue culture plates at 1×10^5 cells/well, maintained in 4N0-500 medium containing 0.9008 g/L glucose (normal glucose), 10% serum, animal-derived growth factors, and 1% penicillin and streptomycin, and incubated at 37°C in a humidified atmosphere of 95% O_2 and 5% CO_2. At sub-confluence, the culture medium was replaced with 4N3-500-S medium containing 0.9008 g/L normal D-glucose, without serum and growth factors. After 24 h, cells were washed twice with phosphate-buffered saline and cultured in serum-free medium challenged with various treatments. Cells were passaged with 0.05% trypsin-EDTA. All studies were performed with cells at passage 3-6.

Treatment

HCFs were grown to sub-confluence in culture media. After being starved in serum-free medium, cardiac fibroblasts were exposed for 24 h to normal glucose conditions, two different HG concentrations (15 and 30 mM), or equimolar osmotic controls (5 mM glucose plus 10 or 25 mM D-mannitol). Four nanograms per milliliter of IL-1β and/or 10 μM eplerenone were added to conditioned media during this period. Incubating times of 6, 12, and 24 h were adopted to evaluate the effects of HG concentration on MMP-2 activity.

Zymography

To assess the gelatinase activity, in-gel zymography was performed using the collected supernatant according to previously described methods (Guo et al., 2005). Conditioned media were collected after various treatments. The samples were normalized to cell protein content and electrophoresed on sodium dodecyl sulfate-polyacrylamide gels containing 1 mg/mL gelatin. The gel was stained with 0.1% Coomassie brilliant blue R-250 in a mixture of methanol, acetic acid, and water. Clear zones against the blue background indicated the presence of gelatinolytic activity. To quantify the gelatinase production, the stained zymograms were scanned in a Dolphin-view image system (Wealtec, USA).

Total mRNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

After a 12-h treatment, total RNA from treated cells was extracted using an Rneasy mini-kit (QIAGEN, Hilden, Germany). First-strand complementary DNA (cDNA) was synthesized with Invitrogen's Thermo Script RT-PCR kit using 20-μL reaction mixture containing 2 μg total RNA and 100 ng random hexamers following manufacturer instructions. Two microliters of the cDNA obtained was allocated for RT-PCR. The upstream and downstream sequences of the MMP-2 primer were 5'-AGATCTGCAAACAGGACATTGTATT-3' and 5'-TTCTTCTTCACCTCATTGTATCT CC-3', respectively, with an amplified fragment length of 401 bp. The reaction conditions were 33 cycles of 94°C for 30 s, 60°C for 60 s, and 72°C for 60 s. The upstream and downstream sequences of the TIMP-2 primer were 5'-GTCAGTGAGAAGGAAGTG GACTCT-3' and 5'-ATGTTCTTCTCTGTGACCCAGTC-3', respectively, with an amplified fragment length of 400 bp. The reaction conditions were 25 cycles of 94°C for 30 s, 60°C for 60 s, and 72°C for 60 s. The upstream and downstream sequences of the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer (internal reference) were 5'-ACATCATCCCT GCCTCTACTGG-3' and 5'-AGTGGTGTCGCTGTTGAAGTC-3', respectively, with an amplified fragment length of 261 bp. The reaction conditions were 30 cycles of 94°C for 30 s, 62°C for 60 s, and 72°C for 60 s. The final densities of the MMP-2 and TIMP-2 bands were expressed relative to the corresponding density of the GAPDH band from the same RNA sample.

Statistical analysis

Data are reported as means ± standard deviation (SD) from four independent experiments. Differences among groups were analyzed for significance by one-way analysis of variance (ANOVA) followed by the unpaired Student t-test. Significance was accepted at $P < 0.05$.

RESULTS

Effects of HG concentrations on MMP-2 activity and mRNA expression

As shown in Figure 1, both 30 mM glucose and D-mannitol (as an osmotic control) significantly increased gelatinolytic activity of MMP-2 in cultured HCFs. Consistent with the gelatinolytic activity of MMP-2, RT-PCR analysis revealed that high concentrations of glucose and D-mannitol enhanced mRNA transcription of MMP-2 after a 12-h incubation (Figure 2). Moreover, the effect of HG on the activity and mRNA expression of MMP-2 was stronger than that of the same concentration of D-mannitol in HCFs. However, compared with normal glucose (5 mM D-glucose, control), 15 mM glucose and D-mannitol had no obvious influence on gelatinolytic activity or mRNA expression of MMP-2. Therefore, 30 mM glucose (HG) was used for further experiments. In contrast, zymography and RT-PCR revealed no effect of HG on MMP-9 activity and mRNA expression in HCFs, respectively (data not shown). Figure 3 shows the time-dependent activity increase of MMP-2 induced by HG. In contrast to the effect of normal glucose, activity of MMP-2 significantly increased after a 12-h incubation in HG. However, the maximal induction of HG on MMP-2 activity occurred at 24 h.
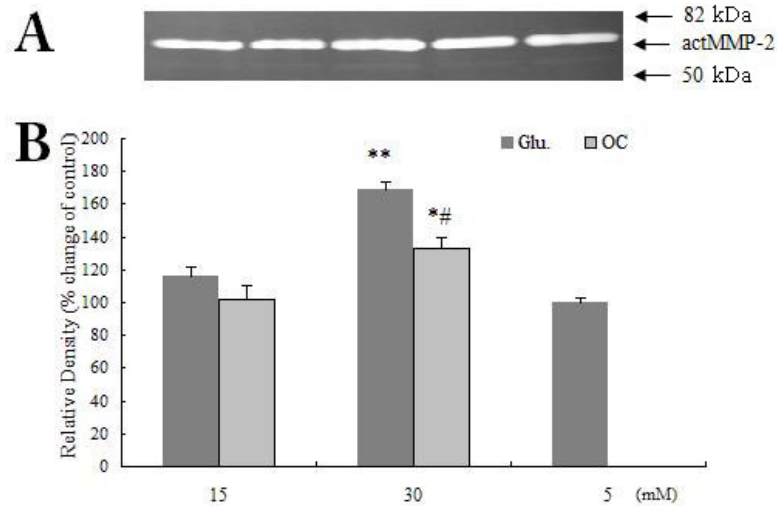
Figure 1. Effects of glucose (Glu.) on MMP-2 activity in cultured human cardiac fibroblasts. At subconfluence, the cultured medium was replaced with without-serum medium for 24 h. Subconfluent cells in without-serum medium were treated with different concentrations of glucose in the sequential order, namely, 5, 15, and 30 mM with proper osmotic controls (OC). After a 24-h glucose incubation, the conditioned media and cell lysate were collected and MMP-2 activity in conditioned media were measured using in-gel zymography. **A.** Representative zymogram demonstrating the changes in MMP-2 activity. **B.** MMP-2 activity, expressed as percentages of 5 mM glucose (normal glucose, control) (N = 4). *P < 0.05 *vs* control; **P < 0.01 *vs* control; #P < 0.05 *vs* 30 mM Glu.
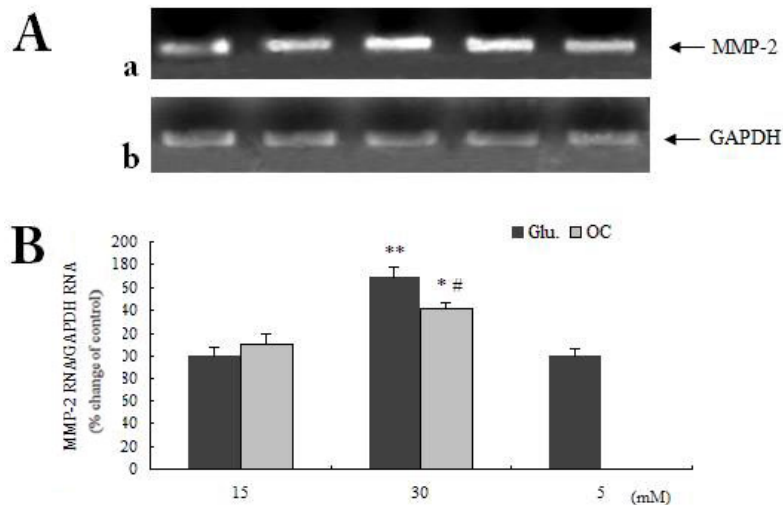

Figure 2. Effects of glucose (Glu.) on MMP-2 mRNA expression in cultured human cardiac fibroblasts. After a 12-h incubation in increasing concentrations of glucose, total RNA was collected and subjected to RT-PCR assay. **A.** (a) is a representative band of PCR products of MMP-2 and amplification of GAPDH. (b) was used as an internal control. **B.** Quantitative results of MMP-2 [firstly normalized for the levels of GAPDH mRNA, then expresses as percentages of the amount of control (normal glucose)] (N = 4). OC = osmotic controls. *P < 0.05 *vs* control; **P < 0.01 *vs* control; #P < 0.05 *vs* 30 mM Glu.
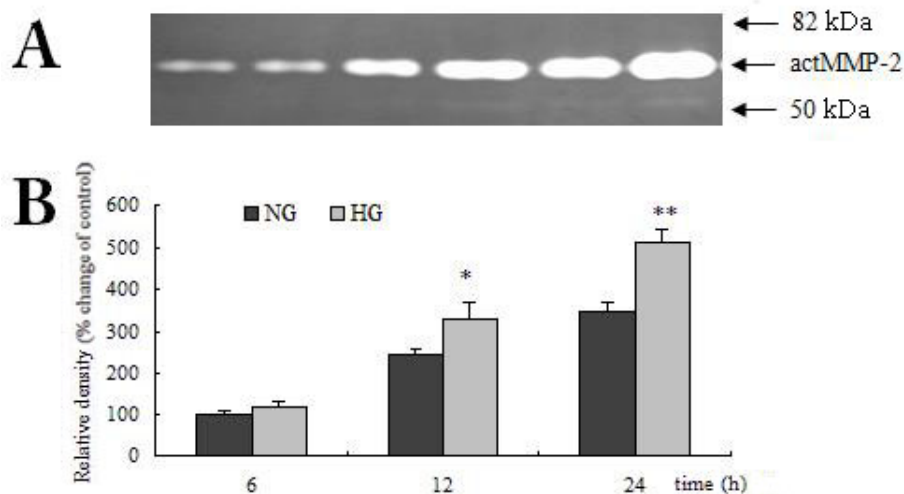
Figure 3. Thirty millimolar of glucose [high glucose (HG)] time-dependently increases MMP-2 activity of cultured human cardiac fibroblasts. **A.** MMP-2 gelatinolytic activities analyzed by zymography. **B.** Histogram representing quantitative results obtained by densitometry of MMP-2 bands shown in Panel A (N = 4). NG = normal glucose. *P < 0.01 *vs* NG (incubating time = 12 h); **P < 0.01 *vs* NG (incubating time = 24 h).

Synergistic effects of HG and IL-1β on MMP-2 activity and expression

We evaluated the effects of HG and IL-1β, alone and in combination, on MMP-2 activity and expression (Figure 4). Although both HG and IL-1β alone increased MMP-2 activities, their combination resulted in an obvious increase in MMP-2 activity compared with the combination of normal glucose and IL-1β (Figure 4A and B). These results demonstrate that HG and IL-1β stimulate MMP-2 activity in HCFs synergistically. Cellular MMP-2 mRNA expression was also analyzed by RT-PCR. Results showed that for HCFs, either HG or IL-1β alone increased the MMP-2 mRNA level after a 12-h incubation. However, the combination of HG and IL-1β led to a maximal increase in the MMP-2 mRNA level (Figure 4C and D). Thus, the increase of MMP-2 mRNA was similar to that of MMP-2 activity (Figure 4).

Inhibition of eplerenone on MMP-2 activity and mRNA expression induced by HG and IL-1β in cultured HCFs

To examine whether eplerenone could reverse HG concentration-induced increases in the MMP-2 activity and mRNA level of HCFs, 10 μM eplerenone was added to HCF culture media. The addition of eplerenone by itself caused a non-significant decrease in MMP-2 activity and mRNA expression, but did block the effects of HG conditions on MMP-2 activity and mRNA expression (Figure 5A-D). A similar effect of eplerenone was observed with respect to the activity and mRNA expression induced by IL-1β for HCFs. As illustrated in Figure 5E-H, the addition of eplerenone to HCF cultures significantly reversed the IL-1β-induced effects on MMP-2 activity and mRNA expression.
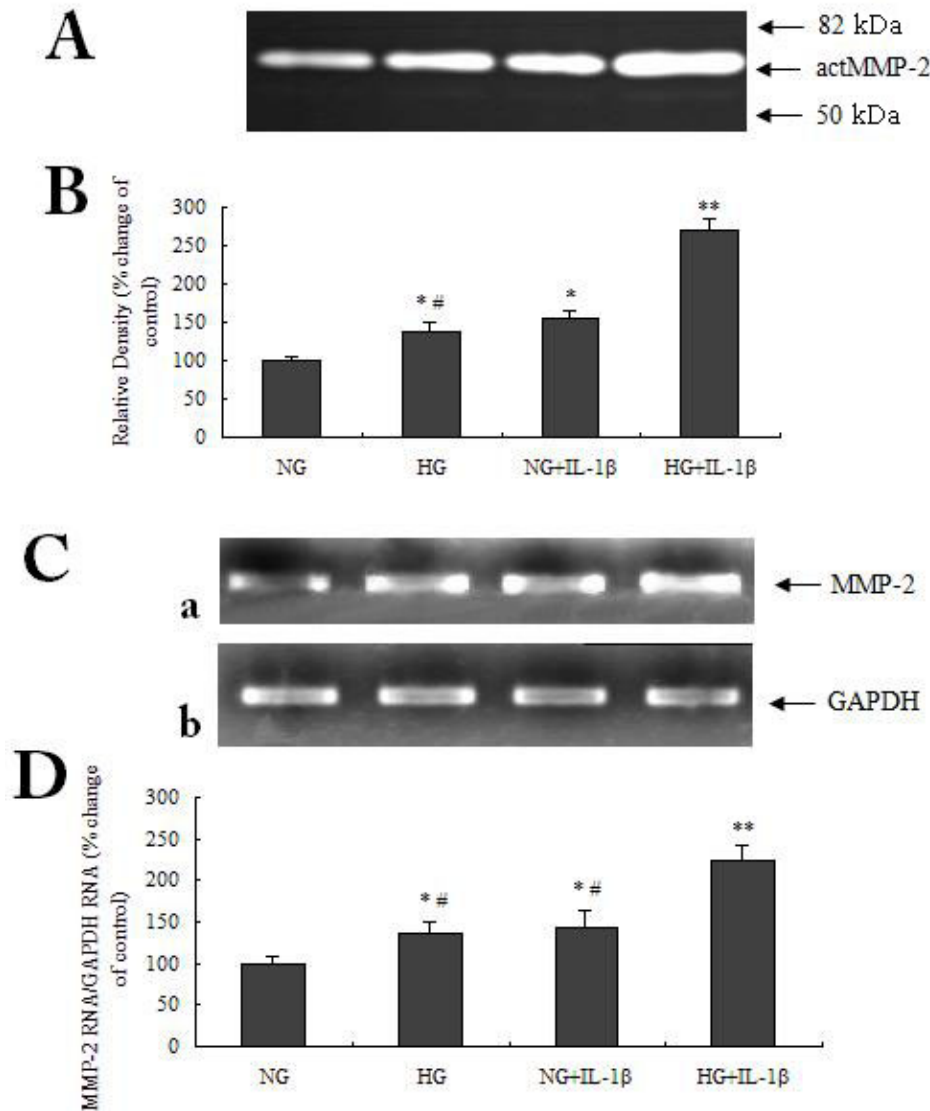
Figure 4. Synergistic effect of high glucose and IL-1β on gelatinolytic activity and mRNA expression of MMP-2 in cultured human fibroblasts. **A.** Activity of MMP-2 by cells exposed to normal or high glucose in the absence or presence of IL-1β. Human cardiac fibroblasts were cultured in normal and high glucose (30 mM) medium and treated with 4 ng/mL IL-1β for 24 h. After the treatment, the conditioned medium was used for in-gel zymography. **B.** Histogram representing quantitative results obtained by densitometry of MMP-2 bands shown in Panel A (N = 4). **C.** RT-PCR analysis of MMP-2 expression by cells exposed to normal or high glucose in the absence or presence of IL-1β. After treatment for 12 h, total RNA was isolated from the cells and used for RT-PCR to analyze MMP-2 [**C** (a)] and GAPDH [**C** (b)] mRNA expression. **D.** Relative MMP-2 mRNA expression calculated by normalization to GADPH mRNA (N = 4). HG = high glucose; NG = normal glucose. *P < 0.05 *vs* control; **P < 0.01 *vs* control; #P < 0.05 *vs* high glucose supplemented with 4 ng/mL IL-1β.
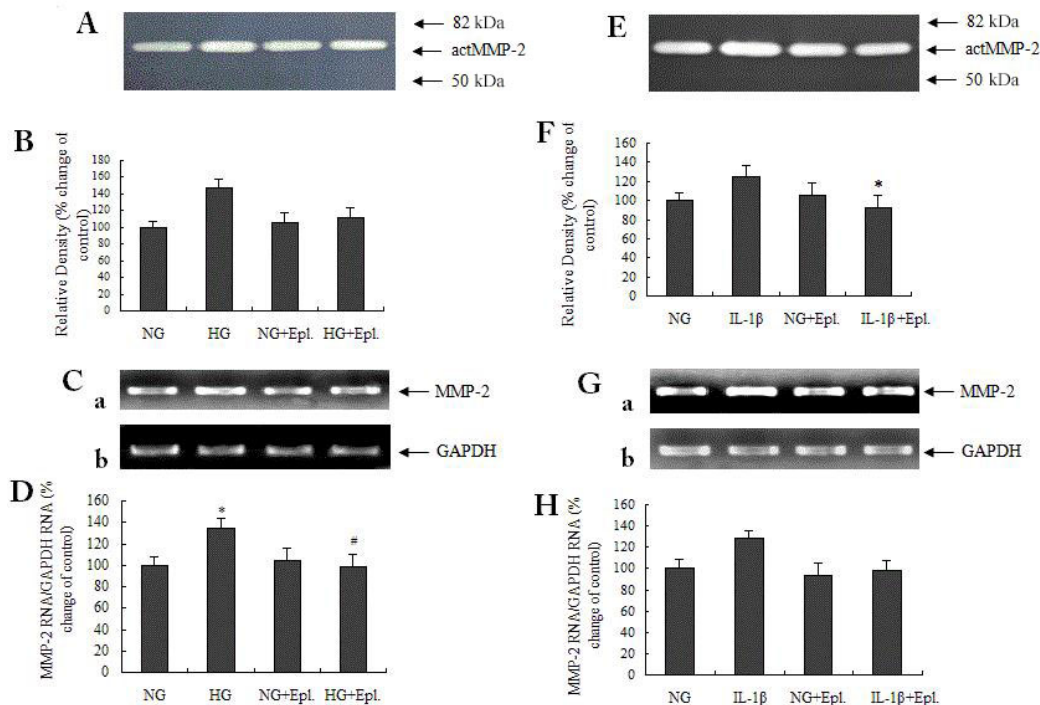
Figure 5. Inhibition of eplerenone (Epl.) on MMP-2 activity and mRNA expression induced by high glucose and IL-1β in cultured human cardiac fibroblasts. Cardiac fibroblasts were treated with high glucose (30 mM) or IL-1β (4 ng/mL) with or without eplerenone (10 µM). **A. E.** Cultured condition medium collected and subjected to in-gel zymography analysis 24 h later. **B. F.** Histograms representing quantitative results obtained by densitometry of MMP-2 bands shown in Panels A and F, respectively (N = 4). **C. G.** RT-PCR analysis of MMP-2 expression. After treatment for 12 h, total RNA was isolated from the cells and used for RT-PCR to analyze MMP-2 (a) and GAPDH (b) mRNA expression. **D. H.** Relative MMP-2 mRNA expression was calculated by normalization to GADPH mRNA (N = 4). HG = high glucose; NG = normal glucose. $*P < 0.05$ vs control; $^{#}P < 0.05$ vs high glucose.

Effects of HG, IL-1β, and eplerenone on TIMP-2 expression in cultured HCFs

In this study, the effect of HG on TIMP-2 expression was also determined. Results showed that HG, IL-1β, and eplerenone had no significant effect on TIMP-2 expression (data not shown).

DISCUSSION

In the present study, a high concentration (30 mM) of glucose was used in experiments based on blood glucose levels and plasma osmolality in normal and diabetic BALB/c mice (Botolin et al., 2005). We found that 24-h HG incubation increased the activity of MMP-2 in a time-dependent manner. Furthermore, we treated cells with different HG levels, including 15 and 40 mM (data not shown), and tested their effects on MMP-2 activity. These results showed

that HG could maximally enhance MMP-2 activity at the 30 mM concentration. Considering that its hyperosmotic effect is a major characteristic of HG, we further treated the cells with equimolar concentrations of D-mannitol, which also enhanced the activation and production of MMP-2. These results suggest that HCFs are actively mediating a hyperosmotic response that modulates the activity and expression of MMP-2 under HG conditions. In addition, other cells such as osteoblasts and human retinal corneal epithelial cells also showed an osmotic response to pathological elevations of MMPs (Li et al., 2004; Botolin and McCabe, 2006). As demonstrated by Smogorzewski et al. (1998) and Howarth et al. (2002), the hyperosmotic pressure induced by HG could increase intracellular Ca^{2+} levels in cells, which could mobilize the release and activation of MMPs such as MMP-2, MMP-9, and others (Jiang et al., 2004). The Ca^{2+}-mediated expression and activation of MMP-2 was shown to be significantly abrogated by the Ca^{2+} channel blocker nifedipine (Yamada et al., 2009). Together, this study suggests that the increase of intracellular Ca^{2+} induced by HG might be, at least partly, involved in the regulation of MMP-2 induced by HG. However, the detailed mechanism remains unclear, and further studies are required to clarify it. Nevertheless, the HG-induced activity of MMP-2 was much higher than that induced by the same concentration of D-mannitol. Thus, it appears that part of the mechanism involved in HG induction of MMP-2 activity is independent of the hyperosmotic response.

Besides the hyperosmotic pressure of HG, chronic exposure of protein to HG also leads to the formation of advanced glycation end-products that are fibrogenic and inflammatory (Matsui et al., 2010). Another study also showed that hyperglycemic myocardial damage was mediated by proinflammatory cytokines (Yu et al., 2011). Furthermore, exposure to HG was found to enhance MMP-1 expression of macrophages in response to inflammatory stimuli (Maldonado et al., 2004). These results suggested that the intervention of inflammatory factors might augment the influence of HG on the activation and expression of MMPs. Our results supported the notion that pro-inflammatory cytokines are important factors for the development of diabetic cardiomyopathy.

The incidence of diabetes associated with cardiovascular disease has increased recently, leading to high morbidity and mortality rates (Mellbin et al., 2010). Therefore, suitable pharmacological treatments that offer protection from diabetic complications are needed, particularly in light of the growing number of patients developing diabetic cardiomyopathy. In the Heart Outcome Prevention Evaluation (HOPE) trial, the angiotensin-converting enzyme inhibitor (ACEI) could not block angiotensin II-dependent adrenal aldosterone secretion after prolonged ACEI therapy (Staessen et al., 1981). Aldosterone receptor antagonism in patients treated with ACEI was shown to reduce mortality in patients with congestive heart failure (Pitt et al., 2001). Diabetes is associated with the development of chronic low-grade inflammation (Biondi-Zoccai et al., 2003; Wellen and Hotamisligil, 2005). As mentioned above, proinflammatory cytokines are important factors for the development of diabetic cardiomyopathy. In order to investigate the effect of aldosterone receptors on diabetic cardiomyopathy, we added eplerenone into conditional medium that contained either HG or IL-1β with HCFs, and found that eplerenone normalized the activity and mRNA expression of HG- or IL-1β-induced MMP-2. Thus, eplerenone might be a favorable option for preventing cardiac ECM degradation in diabetic patients via its influence on MMP-2 activity.

MMPs and TIMPs are present in the myocardium and are both involved in ventricular remodeling (Rastogi et al., 2005). TIMP-2 is the natural inhibitor of MMP-2. In the present

study, no influences of HG, IL-1β, and eplerenone were observed on TIMP-2 mRNA expression, which indicates that the imbalance of the MMP-2/TIMP-2 ratio might regulate by changed MMP-2 expression.

Limitations

In our experiment, we could not elucidate whether HG stimulated MMP-2 activity in cardiac fibroblasts via the mineralocorticoid receptor.

CONCLUSIONS

We demonstrated that exposure of human cardiac fibroblasts to high glucose enhanced MMP-2 activity and expression. We also showed that IL-1β enhanced the priming effect of high glucose on human cardiac fibroblasts. Finally, we demonstrated, for the first time, that eplerenone could normalize the effect of increased MMP-2 activity and expression induced by high glucose and IL-1β. Therefore, eplerenone is suggested as a beneficial therapy against diabetic cardiomyopathy.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

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