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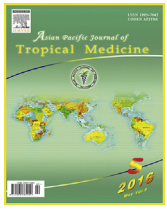

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Influence of hydrogen sulfide on zymogen activation of homocysteine-induced matrix metalloproteinase-2 in H9C2 cardiocytes

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ABSTRACT

Objective: To observe the influence of different concentrations of homocysteine (Hcy) and hydrogen sulfide (H_2S) on the secretion and activation of matrix metalloproteinase-2 (MMP-2) in cardiocytes so as to search for new ways to fight against myocardial tissue fibrosis.

Methods: Cardiocytes H9C2 was cultured *in vitro* and different concentrations of Hcy and H_2S were added for 6-h and 24-h cultivation. MTT cell proliferation assay was applied to test the activation change of cardiocytes H9C2 after affecting by different concentrations of Hcy and H_2S. ELISA and MTT were employed to detect the expression and enzymatic activity of MMP-2.

Results: The H9C2 cell inhibition of activity was more significant with 1 000 μmol/L of Hcy as compared with other concentrations ($P < 0.001$). With 2.5–100.0 μmol/L Hcy and 0.1, 1.0 and 10.0 mmol/L H_2S, the activity of H9C2 did not change significantly ($P > 0.05$). Hcy with concentrations of 10, 50 and 100 μmol/L could increase the quantity of MMP-2 secreted by cardiocytes H9C2, and the interaction strength was concentration-dependent ($P < 0.05$). After interacting with 100 μmol/L of Hcy for 6 h, the zymogen activation effect of MMP-2 was stronger than that of the 2.5–25 μmol/L group ($P < 0.05$). After interacting with Hcy and H_2S (1.0 mmol/L) for 6 h and 24 h, the activation effect of MMP-2 was stronger than those interacted with 10, 25, 50 and 100 μmol/L of Hcy ($P < 0.05$).

Conclusions: Hcy can increase the production of MMP-2 secreted by H9C2 cell and improve its zymogen activation. Besides, the interaction strength is concentration-dependent; while H_2S can up-regulate the activation of MMP-2 and co-promote the activation of MMP-2 with Hcy as well.

1. Introduction

Cardiac extracellular matrix (ECM) is mainly composed of fibrous collagens, elastin, fibronectin and so on, which are responsible for the support and bind of myocardial cells [1]. Types Ⅰ and Ⅲ are the most important collagens for ECM which account on around 80%. For instance, the barrier of collagen synthesis and degradation pathways can lead to myocardial tissue fibrosis, affect the structure and function of heart and also influence the occurrence and development of heart diseases [2,3]. In addition, cardiac interstitial fibrosis will accelerate the change of atrial electrophysiological characteristic. Hence, improvement of atrial tissue fibrosis might be a new way to prevent and treat atrial fibrillation [4]. Matrix metalloproteinase-2 (MMP-2) can degrade most ECM, and adjust the metabolism of ECM with endogenous inhibiting factor (TIMPs) accurately, which make it an important factor for the reconstruction of heart tissue [5–8]. It can be presumed that the regulation mechanism of MMPs/TIMPs is closely related to the prevention and treatment of heart diseases [9]. Some scholars claim that homocysteine (Hcy) can activate the *in-vivo* MMPs zymogen and participate in the pathogenesis of various cardiovascular and cerebrovascular diseases such as hypertension, stroke and peripheral vascular artery atherosclerosis [10]. Hydrogen sulfide (H_2S) possesses a wide physiological regulatory effect on cardiovascular, nervous, digestive and endocrine system and presents a crossed regulating effect with Hcy [11]. The aim of

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this study was to observe the influence of different concentrations of Hcy and H_2S on the secretion and activation of MMP-2 in cardiocytes so as to provide new theoretical basis to prevent and treat myocardial tissue fibrosis.

2. Materials and methods

2.1. Source of cells

The embryonic cardiac H9C2 cells of rats were purchased for the Cell Centre of Shanghai Institutes for Biological Sciences, CAS. BDIX rat embryonic cardiomyocytes offered freely the Pathogen Biology Laboratory by of Shantou University Medical College were from ATCC.

2.2. Reagents and instruments

Reagents and instruments used in this study included MMP-2 quantitative enzyme-linked detection kit, DMEM medium with high glucose (Gibco), 96-well culture plates, 25 cm^2 cell culture flask (Corning), UVP gel scanning system (Bio-RAD), invert microscope, XS105 electronic analytical balance and 756 ultraviolet spectrophotometer (Shanghai Medical Devices).

2.3. Experimental methods

Rat embryonic H9C2 cardiocytes were cultivated serially till 80% of them were blended. They were inoculated on a 96-well culture plates with 100 μL in each hole and an inoculation density of 2×10^4/mL. After 24 h, 0, 10, 50, 100, 500 and 1 000 μmol/L Hcy incubated cells without phenol red medium and 0.1, 1.0 and 10 mmol/L H_2S were added respectively. NaHS with experimental concentrations of Hcy and H_2S but without phenol red medium was used to incubate cells.

2.4. Detected methods for concentrations and activity of MMP-2

After culturing for 6 h and 24 h, MTT cell proliferation assay was applied to test the activation of H9C2 cardiocytes, while ELISA and MTT were employed to detect the expression and enzymatic activity of MMP-2.

2.5. Statistical management

The experimental data were recorded and counted with SPSS13.0. Comparisons between groups were tested by *t*-test and comparisons among groups were analyzed by one-way ANOVA. $P < 0.05$ indicated that the differences were statistically significant and $\alpha = 0.05$ was the inspection level.

3. Results

3.1. Influence of Hcy on cell activity of cardiocytes and synthesis and secretion of MMP-2

The results showed that the cell activity of H9C2 decreased with the increase of the concentrations of Hcy, the cell activity of H9C2 was inhibited significantly when the concentration of Hcy reached 1 000 μmol/L ($P < 0.001$), and the activity of H9C2 showed no statistical significance when the concentration of Hcy was from 2.5 to 100.0 μmol/L ($P > 0.05$) (Table 1). After co-affected and cultivated by 2.5–100.0 μmol/L Hcy and 0.1, 1.0 and 10.0 mmol/L H_2S for 6 h or 24 h, the cell activity of H9C2 showed no significant difference as compared with that of the control group ($P > 0.05$).

Hcy with concentrations of 10, 50 and 100 μmol/L could increase the secretion quantity of MMP-2, and the interaction strength was concentration-dependent ($P < 0.05$) (Table 1).

3.2. Dose-effect relationship of Hcy on activity of MMP-2

The study revealed that Hcy could promote the zymogen activation of MMP-2 significantly ($P < 0.05$). After interacting with 100 μmol/L of Hcy for 6 h, the zymogen activation effect of MMP-2 was stronger than that of the 2.5–25.0 μmol/L group and after interacting with 5–100 μmol/L of Hcy for 24 h, its zymogen activation effect was stronger than that of the 2.5 μmol/L group ($P < 0.05$), which indicated that the promotion effect of Hcy on the zymogen activation of MMP-2 was concentration-dependent (Figures 1 and 2).

3.3. Time-effect relationship of Hcy on zymogen activation of MMP-2

After affected by 5–100 μmol/L for 24 h, the activation of MMP-2 was stronger than that after affected by the same number of Hcy for 6 h ($P < 0.05$) (Figure 3).

3.4. Regulatory effect of H_2S on activity of MMP-2

H_2S with concentrations of 0.1, 1.0 and 10.0 mmol/L could significantly promote the activation of MMP-2 ($P < 0.05$). There was significant difference between the activation effects of 0.1 and 10.0 mmol/L of H_2S and 1.0 mmol/L of H_2S ($P < 0.01$) (Figure 4).

3.5. Corporate regulatory effect of Hcy and H_2S on activity of MMP-2

The results of the co-effect of Hcy and H_2S showed that 1.0 mmol/L of H_2S could promote the activation effect of Hcy significantly (Figure 5); 1.0 and 10.0 mmol/L of H_2S could up-regulate the activation effect of Hcy significantly and $P = 0.002$ after affected by 1.0 mmol/L H_2S for 6 h, while $P = 0.042$ after affecting for 24 h (Figure 6); 1.0 mmol/L of H_2S could raise activation effect of Hcy on the activity of MMP-2 significantly (Figure 7); and 1.0 mmol/L of H_2S could also strengthen the enzyme activation of Hcy (Figure 8).

Table 1
Influence of Hcy on cell activity of cardiocytes and total concentration of MMP-2 secreted by cardiocytes.

Hcy (μmol/L)	OD	Survival rate (%)	MMP-2 (ng/mL)
0	1.65 ± 0.01	100	3.46 ± 0.99
10	1.66 ± 0.03	100	27.08 ± 2.21$^{*\triangle}$
50	1.60 ± 0.06	97	20.25 ± 2.21$^{*\#}$
100	1.60 ± 0.03	96	11.43 ± 1.98$^{*\#\triangle}$
500	1.58 ± 0.03	96	–
1 000	1.33 ± 0.06**	81**	–

Compared with 10 μmol/L, $^{\#}P < 0.05$; compared with 0 μmol/L, $^{*}P < 0.05$; compared with 50 μmol/L, $^{\triangle}P < 0.05$; compared with 0 μmol/L, $^{**}P < 0.001$.

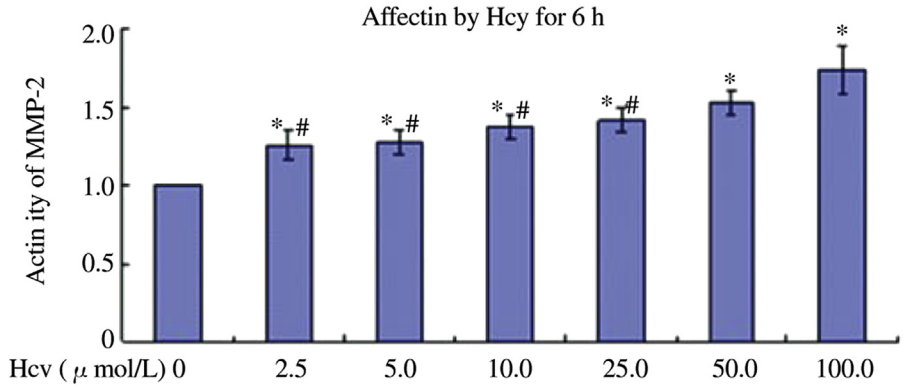

Figure 1. Regulatory effect on activity of MMP-2 after affected by Hcy for 6 h.
Compared with 0 μmol/L, $^{*}P < 0.05$; compared with 100 μmol/L, $^{\#}P < 0.05$.

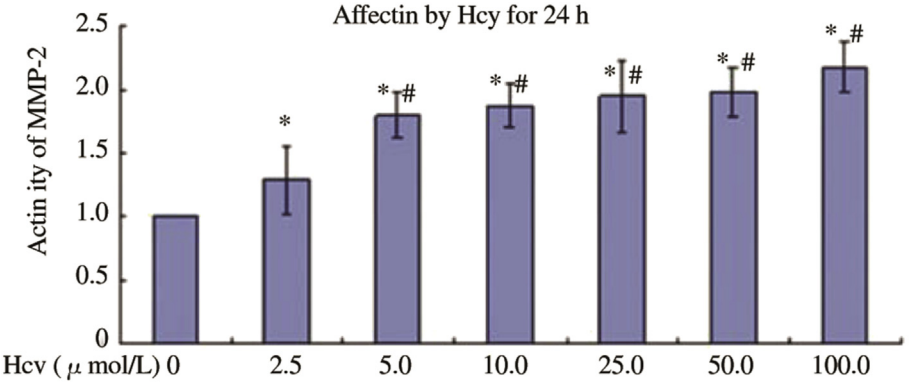

Figure 2. Regulatory effect on activity of MMP-2 after affected by Hcy for 24 h.
Compared with 0 μmol/L, $^{*}P < 0.05$; compared with 2.5 μmol/L, $^{\#}P < 0.05$.

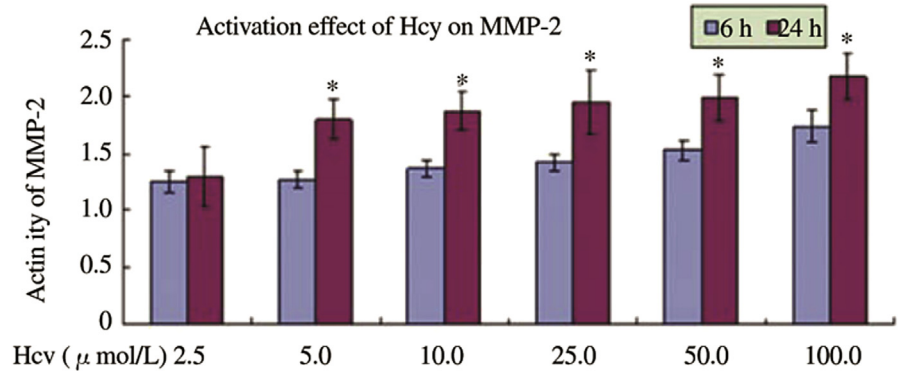

Figure 3. Regulatory effect on activity of MMP-2 after affected by Hcy for 6 h and 24 h.
Compared between affecting for 6 h and 24 h with the same concentration; $^{*}P < 0.05$.

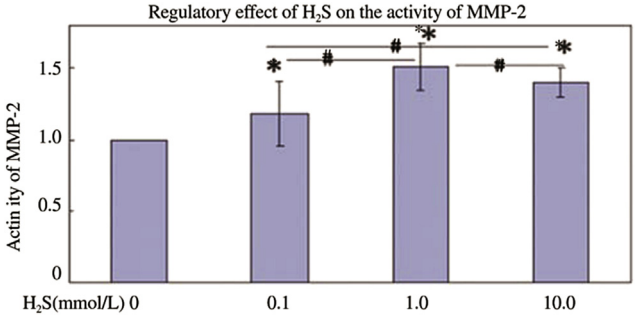

Figure 4. Regulatory effect on activity of MMP-2 after affected by H_2S for 24 h.
Compared with 0 mmol/L, $^{*}P < 0.05$; compared between groups, $^{\#}P < 0.01$.

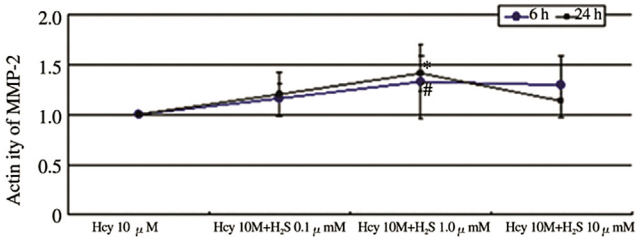

Figure 5. Activity of MMP-2 after co-affected by 10 μmol/L Hcy and H_2S for 6 h and 24 h.
Compared with 10 μmol/L Hcy for 6 h, $^{\#}P < 0.01$; compared with 10 μmol/L Hcy for 24 h, $^{*}P < 0.05$.

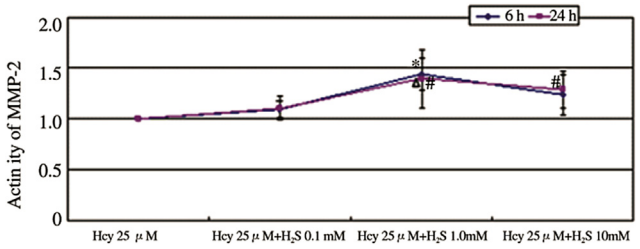

Figure 6. Activity of MMP-2 after co-affected by 25 μmol/L Hcy and H_2S for 6 h and 24 h.
Compared with 25 μmol/L Hcy for 6 h, $^{*}P < 0.01$; compared with 25 μmol/L Hcy for 24 h, $^{\#}P < 0.05$; compared with 0.1 mmol/L of Hcy + H_2S, $^{\triangle}P < 0.05$.

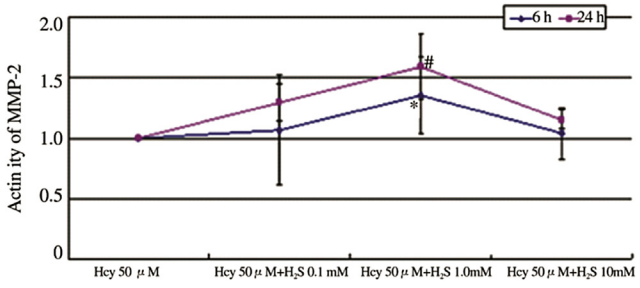

Figure 7. Activity of MMP-2 after co-affected by 50 μmol/L Hcy and H_2S for 6 h and 24 h.
Compared with 50 μmol/L Hcy for 6 h, $^{*}P < 0.05$; compared with 50 μmol/L Hcy for 24 h, $^{\#}P < 0.01$.

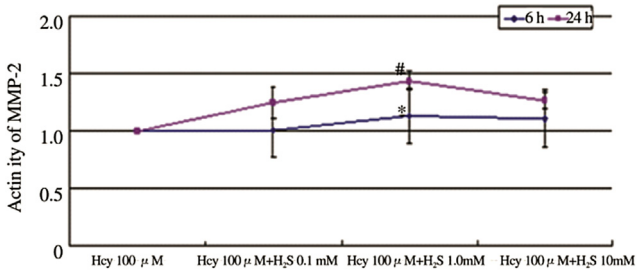

Figure 8. Activity of MMP-2 after co-affected by 100 μmol/L Hcy and H_2S for 6 h and 24 h.
Compared with 100 μmol/L Hcy for 6 h, $^{*}P < 0.05$; compared with 100 μmol/L Hcy for 24 h, $^{\#}P < 0.01$.

4. Discussion

Recently, epidemiological studies have shown that Hcy is closely related to the incidence and development of diseases, such as hypertension, atrial fibrillation, stoke, dementia and so on, and also the abnormal increase of blood concentration of Hcy is an independent risk factor of cardia-cerebrovascular disease [11–13]. The latest *in vitro* studies have demonstrated that Hcy can synthesize H_2S directly by catalysis of CSE enzyme. Although the synthesized H_2S only represents a small part, it can increase with the raise of the concentration of Hcy, and the synthesis of H_2S can increase significantly at a super high concentration of Hcy [14]. H_2S has active sulfhydryl. Therefore, the key point of this study is that whether the enzyme activity of MMP-2 cardiocytes has a regulatory effect.

The activity imbalance between MMPs and TIMPs is a factor causing atrial tissue fibrosis [15]. At present, 28 kinds of MMPs have been discovered. Among them, MMP-2 and MMP-9 all belong to gelatinase and participate in the pathogenesis of many cardiovascular diseases [16]. Some scholars hold the idea that the abnormal of the activity of MMP-2 is the main risk factor of atrial fibrillation [17–20]. Some other scholars insist that Hcy can stimulate the zymogen activation of MMP-2 and facilitate the activation of MMP-2 in the ventricular tissues of rats effectively [21]. There are also researches revealing that Hcy possesses a toxic effect on H9C2 cardiocytes [22]. For example, it can accelerate the apoptosis of H9C2 cardiocytes or inhibit their growth activity evidently. Hcy can obviously promote the apoptosis of H9C2 at a super high concentration of 2.73 mmol/L and 100 and 1 100 μmol/L of Hcy can even lead to reversible changes of the ATP value, mitochondrial transmembrane potential and epicyte of H9C2 cardiocytes. Hence, the effect of Hcy on the activity of cardiocytes was investigated in this study in the first place. The results of MTT showed that the survival rate of H9C2 cardiocytes decreased with the increase of the concentration of Hcy. When the concentration of Hcy reached 1 000 μmol/L, the activity of H9C2 cardiocytes was inhibited significantly ($P < 0.001$), which was identical with the reported ones [23]. In this study, the activity of H9C2 cardiocytes was not influenced significantly when affected by 2.5–100 μmol/L Hcy, which implied that it could be used continuously and safely in the subsequent experiences. In this study, the effect of Hcy on the expression and zymogen activation of MMP-2 of H9C2 cardiocytes was also observed. The results demonstrated that being affected by 0–100 μmol/L of Hcy for 24 h could stimulate the synthesis and activity of MMP-2 cardiocytes in a concentration- and time-dependent manner, which implied that Hcy participate in the reconstruction of heart tissues by inducing the expression and activation of MMP-2 [24]. NaHS was applied as the exogenous donor for H_2S in this study and three concentration groups (0.1, 1.0 and 10.0 mmol/L) were established. The results showed that H_2S with the above concentrations had no significantly influence on the activity of H9C2 cardiocytes, while after acting for 24 h, it promoted the activation of MMP-2 significantly and presented as a concentration-dependent manner. After that, H_2S with the above concentrations were added into the Hcy group, the results showed no changes of the cell activity. Moreover, the study results also showed that Hcy + H_2S synergistically facilitate the activity of MMP-2 positively after acting for 6 h and 24 h, but the time-dependent manner was not found.

The results of this study manifest that Hcy could increase the production of MMP-2 secreted by H9C2 cells and improve its zymogen activation. Besides, the interaction strength is concentration-dependent; while H_2S could up-regulate the activation of MMP-2 and co-promote the activation of MMP-2 with Hcy as well.

Conflict of interest statement

We declare that we have no conflict of interest.

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