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Effects of emodin and irbesartan on ventricular fibrosis in Goldblatt hypertensive rats

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Left ventricular (LV) fibrosis is one of the most prominent pathophysiological results of hypertension. We initiated this study to investigate the effects and mechanisms of emodin and its combination with irbesartan on LV fibrosis in Goldblatt (2K1C) hypertensive rats. Goldblatt hypertension rats were prepared by two kidney one clip (2K1C) operations and then treated with either emodin, irbesartan or their combination. As a result, the systolic blood pressure (SBP) and the left ventricular mass index (LVMI) increased significantly ($P \leq 0.05$) in all 2K1C rats. After drugs treatment, irbesartan and the drug combination remarkably decreased SBP, LVMI, contents of angiotensinII (AngII), hydroxyproline and collagen, the mRNA and protein expression levels of matrix metalloproteinase-2 (MMP-2) and tissue inhibitor of metalloproteinase-2 (TIMP-2) ($P \leq 0.05$). As for the emodin, LVMI, contents of hydroxyproline and collagen, and MMP-2 and TIMP-2 expression were found to decrease significantly; however, the SBP and AngII contents stayed stable within certain extent. Therefore, emodin, irbesartan or two drugs together can potentially inhibit the ventricular fibrosis in Goldblatt hypertensive rats by reducing MMP-2 and TIMP-2 expression. Furthermore, the combination of these two drugs may provide a better anti-fibrosis effect than the single application.

1. Introduction

Hypertension can cause serious complications such as heart failure, stroke and kidney failure, leading to high morbidity and mortality. Left ventricular (LV) remodeling is one of the most prominent pathophysiological results of hypertension, and is characterized as pathological hypertrophy of cardiac myocytes and abnormal increases of matrix collagen synthesis, concentration or volume fraction (Rizzi et al. 2013), which could eventually result in pathologic fibrosis. During this process, matrix metalloproteinase-2 (MMP-2) is a potent regulator of collagen metabolism (Visse and Nagase 2003), and promote myocardial fibrosis by increasing collagen synthesis (Bergman et al. 2007; Kassiri et al. 2009). Besides, tissue inhibitor of metalloproteinase-2 (TIMP-2), an endogenous antagonist of MMP-2, may inhibit MMP-2 activity and attenuate myocardial fibrogenesis (Kandalam et al. 2011). The interaction of the two proteins plays a very key role in cardiac structure and function, and they are considered as the important factors for myocardial fibrosis and cardiac remodeling.

The role of hypertension in myocardial fibrogenesis mechanism remains largely unknown. Currently, some stimuli are believed to participate in the pathological lesion, such as over-expression of the renin-angiotensin aldosterone system (RAAS), oxidative stress activation, and excessive MMPs activities (Wynn 2008). Among them, interactions between the RAAS and MMPs have been well documented. AngiotensinII (AngII) is considered to be an important regulator of fibrosis (Zile et al. 2011; Li et al. 2011; Weber et al. 2013), and significantly up-regulates expression and activity of MMP-2, which further induces and promotes the cardiac fibrosis (Striker et al. 2008; Jiménez et al. 2009; Saygili et al. 2009).

Hypertension can impair diastolic function, worsen LV compliance and increase arterial stiffness. Recent studies have shown that the continuous stretch stimulation of vascular smooth muscle cells up-regulated the expression of angiotensin type I receptor (ATR_1) and enhanced the activity of AngII (Liu et al. 2010). The diastolic dysfunction caused by hypertension is still difficult to treat. At present, ATR_1 blockers (ARBs) like irbesartan are effectively used to control hypertension, inhibit left ventricular hypertrophy according to the SILVHIA study project. However, occurrence and development of tissue fibrosis are not confined to the RAAS, and hence ARBs could only partially prohibit formation of fibrosis. Additionally, the risk and side effects of these drugs also restricts their clinical use (Symvoulakis et al. 2007).

Dried root of *Polygonatum multiflorum*, well known as Radix Polygoni Multiflori or Hersouwu, is a popular traditional Chinese medicine (Zhao 2004). This herb has been used commonly to treat diseases associated with aging and possesses antitumor, hypocholesterolemic, vasorelaxant and antioxidative effects *etc*. (Zhang et al. 1983; Xiao et al. 1993; Chiu et al. 2002). Its main active constituents include anthraquinones, stilbenes, phenolic compounds and their glycosides (Zheng et al. 1997). Emodin is considered as the representative anthraquinone in *P. multiflorum* (Ye et al. 2007), and it can also be isolated from another important traditional herbal medicine, rhubarb (Huang et al. 2007). Emodin has been reported to improve the brain

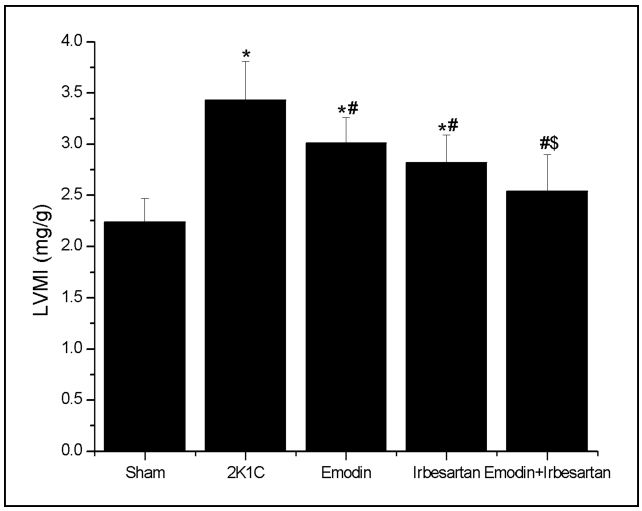

Fig. 1: LVMI values among five groups at end of the 12th week. Note: $^{*}P<0.05$ versus sham; $^{\#}P<0.05$ versus 2K1C; $^{\$}P<0.05$ versus 2K1C/emodin and 2K1C/irbesartan.

disturbances induced by some cerebral injuries, inhibit lipid peroxidation in the brain homogenates and furthermore ameliorate cycloheximide-induced impairment of memory consolidation (Sato et al. 1992; Gu et al. 2000; Lu et al. 2007). In addition, this compound was found to strongly inhibit herpes simplex viruses *in vitro* and *in vivo* (Xiong et al. 2011). Recent pharmacological studies revealed emodin to have multiple bio-activities such as inhibiting the activity of tyrosine kinase receptor and down-regulating the expression of TNF-α and TGF-β1 (Yim et al. 1999; Heo et al. 2008). Therefore, it has been widely used for its anti-inflammatory, anti-tumor and anti-hypertension effects. More interestingly, it is revealed that emodin may contribute to inhibit the fibrosis of pancreas and lives (Zhan et al. 2004; Wang et al. 2007). However, the effect of emodin on myocardial fibrosis, and the possible synergetic action of emodin in combination with irbesartan remains unknown. In this study, we examined whether emodin alone or the combination with irbesartan could inhibit myocardial fibrosis based on the Goldblatt (2K1C) hypertensive rat model.

2. Investigations and results

2.1. Effects of emodin and irbesartan on rat SBP

Four rats died accidently during the experiments including two in the 2K1C group, one in the 2K1C/irbesartan group and one in the 2K1C/E + I group. SBP in all 2K1C rats increased significantly by ~ 50 and 60% ($P \leq 0.05$) compared with that of the sham group after four and eight weeks of operation, indicating the Goldblatt hypertension models were successfully established. After treatment with emodin, irbesartan or both drugs respectively, irbesartan alone and its combination with emodin could strongly decrease the SBP in Goldblatt rats ($P<0.05$), but emodin alone did not influence SBP.

2.2. Effects of emodin and irbesartan on rat LV mass

After 12 weeks of the operation, LVMI of the 2K1C rats was significantly higher than that of sham groups which showed occurrence of myocardial remodeling and LV fibriosis. However, emodin and irbesartan enable their LVMI to decrease greatly ($P<0.05$), and the inhibitory effect was significantly enhanced after combined treatment with both drugs, and even the LVMI was similar to the level of sham rats (Fig. 1).

Table: Effects of emodin and irbesartan on the levels of AngII, hydroxyproline and collagen in hypertensive rats ($\overline{x} \pm s$)

Group	AngII (ng/g)	Hydroxyproline (μg/mg)	Collagen (μg/mg)
Sham	53.67 ± 12.90	0.37 ± 0.10	3.08 ± 0.79
2K1C	$100.54 \pm 20.9^{*}$	$0.61 \pm 0.30^{*}$	$5.01 \pm 2.47^{*}$
2K1C/Emodin	$90.83 \pm 12.60^{*}$	$0.43 \pm 0.15^{\#}$	$3.56 \pm 1.24^{\#}$
2K1C/Irbesartan	$60.54 \pm 11.16^{\#\$}$	$0.41 \pm 0.15^{\#}$	$3.38 \pm 1.22^{\#}$
2K1C/E + I	$56.63 \pm 11.20^{\#\$}$	$0.38 \pm 0.10^{\#}$	$3.12 \pm 0.80^{\#}$

Note: $^{*}P<0.05$ versus sham; $^{\#}P<0.05$ versus 2K1C; $^{\$}P<0.05$ versus 2K1C/emodin and 2K1C/irbesartan.

2.3. Effects of emodin and irbesartan on the contents of AngII, hydroxyproline and collagen in rat LV tissues

At the eighth week after the 2K1C operation, the Goldblatt hypertension formed and then the contents of AngII, hydroxyproline and collagen in the 2K1C group were determined. The results revealed that these indices were significantly higher than those in the sham group ($P<0.05$) (Table), which demonstrated the occurrence and enhancement of the RAAS activity and ventricular fibriosis.

The pharmacological experiment found that the contents of the above indices reached 90.83 ± 12.60, 60.54 ± 11.16 and 56.63 ± 11.20 in the 2K1C/Emodin, 2K1C/Irbesartan and 2K1C/E + I group, respectively. Irbesartan and its combination with emodin could significantly decrease the AngII levels ($P<0.05$), but emodin had no significant effect in spite of a depressing tendency. The three treatment patterns had identical effectiveness and they all significantly decreased ($P<0.05$) the levels of hydroxyproline and collagen (Table).

2.4. Effects of emodin and irbesartan on the expression of MMP-2 and TIMP-2 mRNA

The expression of MMP-2 and TIMP-2 mRNA in the 2K1C group was significantly enhanced ($P<0.05$) compared with the sham group (Table), but significantly decreased when Goldblatt rats were treated by emodin, irbesartan, and the combination of both drugs ($P<0.05$). More importantly, there was no significant difference between the 2K1C/E + I group and the sham group ($P>0.05$) (Fig. 2, 3).

2.5. Expression of MMP-2 and TIMP-2 proteins

The expression of MMP-2 and TIMP-2 proteins was detected by Western blot, suggesting that the target proteins from the 2K1C group had higher expression than those of the sham rats

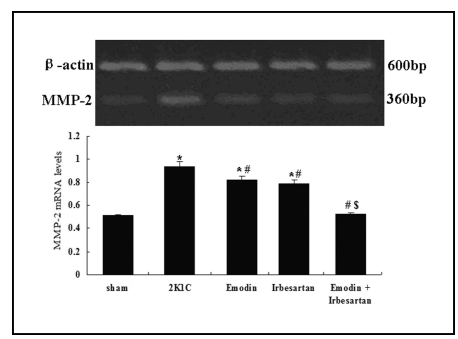

Fig. 2: Effects of emodin and irbesartan on expression of MMP-2 mRNA. Note: The data were generated from optical density measurements of individual bands from RT-PCR and normalized to β-actin. $^{*}P<0.05$ versus sham; $^{\#}P<0.05$ versus 2K1C; $^{\$}P<0.05$ versus 2K1C/emodin and 2K1C/irbesartan.

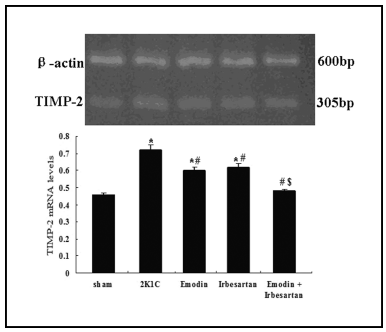

Fig. 3: Effects of emodin and irbesartan on expression of TIMP-2 mRNA. Note: The data were generated from optical density measurements of individual bands from RT-PCR and normalized to β-actin. $^{*}P < 0.05$ versus sham; $^{\#}P < 0.05$ versus 2K1C; $^{\$}P < 0.05$ versus 2K1C/emodin and 2K1C/irbesartan.

($P < 0.05$). Meanwhile, MMP-2 and TIMP-2 expression was acutely down-regulated among the three drug groups in comparison with the 2K1C model group. Additionally, no significant difference was observed between the 2K1C/E + I group and the sham group ($P > 0.05$) (Fig. 4).

3. Discussion

In the 2K1C group, a significant increase of the hydroxyproline and collagen contents and the LV mass confirmed the form and worsening of ventricular fibriosis during the development of Goldblatt hypertension. The strong activation of AngII, MMP-2 and TIMP-2 further revealed the close relationship between RAAS activity and ventricular fibriosis.

Emodin is a natural bioactive product from traditional herbal medicines such as Radix Polygoni Multiflori and Rhubarb, and possesses extensive pharmacological actions including antibiosis, anti-tumor and protecting brain function (Ma et al. 2013). However, our studies, for the first time, revealed the protective effects of emodin on cardiac fibrosis in Goldblatt hypertensive rats. Although the SBP and AngII content in the emodin group did not change significantly compared with the 2K1C group, but the LV mass and the expression of hydroxyproline, collagen, MMP-2 and TIMP-2 all decreased significantly, which suggested that fibrosis was efficiently prohibited. A previous report had confirmed that emodin could down-regulate the expression of transforming growth factor-β1 (TGF-β1) and connective tissue growth factor (CTGF), which could directly inhibit the expression of MMP-2 and TIMP-2 (Chen et al. 2012).

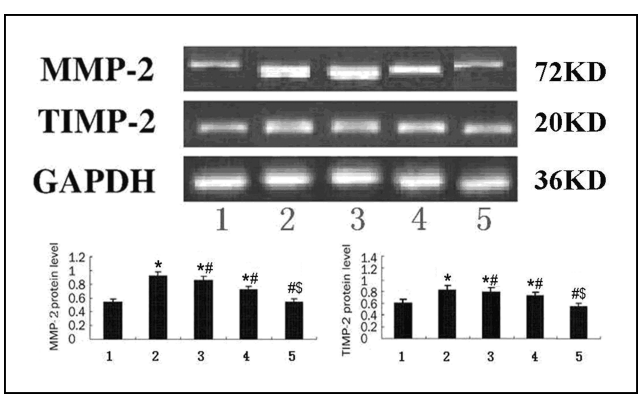

Fig. 4: Effects of emodin and irbesartan on expressions of MMP-2 and TIMP-2 protein in hypertensive rats. Note: Lane 1: sham group; Lane 2: 2K1C group; Lane 3: 2K1C/emodin group; Lane 4: 2K1C/irbesartan group; Lane 5: 2K1C/E + I group. The data were generated from optical density measurements of individual bands from RT-PCR and normalized to β-actin. $^{*}P < 0.05$ versus sham; $^{\#}P < 0.05$ versus 2K1C; $^{\$}P < 0.05$ versus 2K1C/emodin and 2K1C/irbesartan.

Hence, it is indicated that emodin prohibited fibrosis by the TGF-β1/CTGF/MMP-2/TIMP-2 pathway instead of the RAAS system, and might improve LV compliance by blocking the signaling pathway (Kuo et al. 2001).

Irbesartan could significantly decrease the expression of AngII, which is similar to Mizuno and Takai's reports (Barlucchi et al. 2001; Takai et al. 2005). The result showed that the process of LV fibrosis was closely related to AngII from local tissues and irbesartan could significantly improve myocardial fibrosis by prohibiting the function of local AngII (Mirotsou et al. 2011). In the 2K1C/E + I group, the combination of emodin plus irbesartan enable the SBP, LVMI, AngII, MMP-2 and TIMP-2 all had further decreased in Goldblatt rats, suggesting that the combination of both drugs might have a synergistic effect on attenuating LV fibrosis through the AngII-MMP-2/TIMP-2 signal pathway. Though the combination of both drugs had not significant difference from the single drug application about the effects of hydroxyproline and collagen, a depressed tendency was still observed and its stronger inhibitory action on the LV fibrosis could be confirmed together with the LVMI index.

The pathogenesis of LV fibrosis in hypertension is not yet completely clear, and there is no effective treatment method at present. Thus, it is important to extend this research to find and demonstrate a novel pharmacological function of emodin, which is a promising agent in the clinical therapy of cardiovascular diseases associated with myocardial fibrosis.

4. Experimental

4.1. Animals, material, and experimental design

To assess the effects of emodin and its combination with irbesartan as the preventive agents against Goldblatt hypertension, 48 male Sprague-Dawley rats after two kidney one clip (2K1C) operations with anesthetization using 3% pentobarbital sodium to obtain hypertension (Guan et al. 1992), were randomly divided into 4 groups. The animals were further treated with either saline (2K1C group, n = 12), emodin (2K1C/Emodin group, n = 12), irbesartan (2K1C/Irbesartan group, n = 12), or a combination of emodin and irbesartan (2K1C/E + I group, n = 12). In parallel, in the sham group (n = 12) the sham-clipped operation was carried out and the animals were treated with saline but did not receive the drugs. The study was approved by the ethics committee of Guangdong Medical College.

After 4 weeks of operation, systolic blood pressure (SBP) of these rats was measured using the Tail cuff method with ALC-NIBP blood pressure monitor (Alcott Biotech CO., LTD, Shanghai, China) (Li et al. 2012). All the 2K1C rats had higher SBP (≥ 150 mmHg) than basic blood pressure. All rats received consecutive treatments by gavage administration for eight weeks, and the body weight (BW) of each rat was measured at last.

4.2. High-frequency echocardiography

The LV mass of the rats from different experimental groups were detected by high frequency ultrasonography. The rats were anesthetized with 3% pentobarbital sodium and then analyzed on Vivid 7 colored Doppler ultrasonic diagnostic equipment with a 7 MHz probe (GE, USA). Based on the measure of M hypercurve, mean values were obtained from the five consecutive cardiac cycles. The echocardiography indices in this study were shown as following: the left ventricular end diastolic diameter (LVEDD), the left ventricular end systolic diameter (LVESD) and the end diastolic left ventricular posterior wall thickness (LVPWTd). Finally the left ventricular mass index (LVMI) was calculated according to the formula: $LVMI = 1.05[(LVEDD + 2\,LVPWTd)^3 - LVEDD^3]/BW$.

4.3. Measurement of hydroxyproline, collagen and AngII in LV tissues

At the end of the treatment period, rats were euthanized by cervical dislocation. The tissues were prepared as the previously described (Ju et al. 2012). Briefly, fresh LV apical tissues (150 mg/animal) were collected and immediately homogenized in 2 ml 0.5 mol/L glacial acetic acid. The mixtures were divided into two parts, and one part was dried at 60 °C overnight, and then hydrolyzed at 110 °C for 12 h with 6N hydrochloric acid. The mixtures were treated using chloramine T solution and Ehrlich's solution (Jiancheng Bioengineering Institute, Nanjing, China) at 75 °C for 15 min. The absorbance was recorded at λ560 nm with a spectrophotometer

(NanoDrop ND-1000 Spectrophotometer, NanoDrop Products, Wilmington, USA), and was converted into the content of hydroxyproline. Meanwhile, the content of collagen was 8-fold that of hydroxyproline (Switzer and Summer 1971).

The other part of LV tissue homogenate was boiled and centrifuged (3000 rpm for 10 min). The levels of AngII in the supernatants were detected by a radiommunoassay kit according to the manufacture's instruction (North Biotechnology Institutes, Beijing, China).

4.4. Expression analysis of MMP-2 and TIMP-2 mRNA by RT-PCR

Total RNA was extracted from LV tissue using a Trizol isolation kit (Invitrogen, Shanghai, China). The target genes MMP-2, TIMP-2 and β-actin were cloned with Primescript one-step reverse transcriptase-polymerase chain reaction (RT-PCR) kit ver2 (Takara Biotechnology Co., Ltd., Dalian, China), in which β-actin mRNA acted as an internal standard. Their specific primers were: MMP-2: 5'-TTC TTC GCA GGG AAT GAG-3' and 5'-CTT CCA AAC TTC ACG CTCT-3'; TIMP-2: 5'-AAG GAC CTG ACA AGG ACA TCG-3' and 5'-CCA TCC AGA GGC ACT CAT CC-3'; β-actin: 5'-GGT ATG GGT CAG AAG GAC TCC-3' and 5'-ACC GTC AGG CAG CTC ATA GCT-3'. The amplification reactions were performed for 30 cycles consisting of 94 °C for 2 min, 52 °C for 15 s, 72 °C for 1 min. The amplified products and the 150 bp DNA ladder were electrophoresized in agarose gel. The density of the products was detected by Photoshop7.0 software and the data were normalized by β-actin.

4.5. Expression analysis of MMP-2 and TIMP-2 protein by western-blot

The equal amounts of protein extracted from different groups of LV apical tissues were separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and then was electrically transferred to polyvinylidene difluoride membranes. After blocking by 5% skim milk, the membranes were incubated with rabbit anti-rat MMP-2 antibody (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-rat TIMP-2 antibody (1:1000; Santa Cruz Biotechnology) and GAPDH (1:1000; Abcam, HK) overnight at 4 °C. After washing three times, the membranes were incubated with horseradish peroxidase conjugated secondary antibody (Santa Cruz Biotechnology). At last, the signals were detected by enhanced chemiluminescence and analyzed using Kodak 1D 3.5 imaging software (Eastman Kodak, Rochester, NY). All the protein expressions were normalized to GAPDH content.

4.6. Statistical analysis

Statistical analyses were performed by SPSS 17.0 for Windows (SPSS Inc, Chicago, IL). Data were expressed as means ± SD ($\bar{x} \pm s$). One-way ANOVA followed by Student's t test for multiple comparisons was used to compare the difference in all variables between groups. $P < 0.05$ was considered as statistical significance.

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