Baicalein and wogonin inhibit collagen deposition in SHR and WKY cardiac fibroblast cultures

Ebenezer KC Kong1,2, Yu Huang1,3, John E Sanderson1,2, Kar-Bik Chan2, Shan Yu1,2 & Cheuk-Man Yu1,2,*
1Institute of Vascular Medicine, Li Ka Shing Institute of Health Sciences, 2Division of Cardiology, Department of Medicine and Therapeutics, Prince of Wales Hospital, The Chinese University of Hong Kong, 3Department of Physiology, The Chinese University of Hong Kong, Hong Kong

In order to demonstrate the potential therapeutic effect of two flavonoids, Baicalein and Wogonin, on suppression of pathological myocardial fibrosis in hypertension, we investigated their in vitro effects on collagen expression in primary cultured cardiac fibroblasts isolated from neonatal normotensive (WKY) and hypertensive (SHR) rats. Our results showed that over-expression of collagen mRNA and protein induced in cardiac fibroblasts by angiotensin (AngII) could be attenuated significantly by both flavonoids at an optimal dosage (30 μM; P < 0.01). Results of immunoblots showed that expression of 12-LO level, p-ERK/ERK ratio and MMP-9 in AngII-stimulated SHR cardiac fibroblasts were significantly down-regulated by both flavonoids. Our results show that both Baicalein and Wogonin can suppress collagen deposition in AngII-stimulated SHR and WKY cardiac fibroblasts. [BMB reports 2010; 43(4): 297-303]

INTRODUCTION

Myocardial fibrosis is a pathological condition which is commonly observed in nearly all major cardiac diseases including hypertension, cardiomyopathy and heart failure, as well as cardiac remodeling after myocardial infarction (1). In hypertensive hearts, a cardinal feature is the deposition of interstitial fibrosis between cardiomyocytes and perivascular fibrosis surrounding coronary arterioles (2). The development of left ventricular hypertrophy in hypertension is also characterized by both cardiomyocyte hypertrophy and interstitial fibrosis (3). Interstitial collagen is the major structural component of the extracellular matrix (ECM), among which fibrillar collagens, COL-I and COL-III, are the main types in the heart (4). Increase in COL-I, and to a less extent COL-III, have been shown to be major determinants of increased ventricular stiffness in hypertension leading to left ventricular diastolic dysfunction (5). With the progression of the disease it will predispose to the development of complications such as diastolic heart failure or heart failure with a normal ejection fraction (6).

Several studies have suggested that collagen synthesis correlated positively, with the activation of MAPKs, including extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK) and p38, which are implicated in cardiac fibrosis (7). 12-Lipoxygenase (12-LO) also is one of the factors that controls collagen degradation and growth of cardiac fibroblasts (8). 12-LO catalyzes arachidonic acid to 12-hydroperoxyeicosatetraenoic acid (12-HPETE), a 12-hydroperoxy fatty acid, which is then reduced by glutathioneperoxidase to 12-hydroxyeicosatetraenoic acid (12-HETE). 12-HETE also can induce ERK1 (p44 MAPK)/ERK2 (p42 MAPK) phosphorylation, thus initiate TIMP-2 synthesis and inhibit the collagen degradation (9). Besides these, matrix metalloproteinase (MMPs), especially the MMP9, are involved in collagen homeostasis (10, 11).

Currently, treatment for hypertension targets primarily on the blood pressure (12). As myocardial fibrosis plays a pivotal role in the pathophysiological mechanism in cardiac dysfunction, antifibrotic pharmacological therapy is another potentially useful strategy. Previous studies have found that drugs acting on the renin-angiotensin-aldosterone system may have anti-fibrotic effects in the myocardium (13, 14) and they have become one of the most commonly prescribed anti-fibrotic agents.

Scutellaria baicalensis Georgi (or Huang Qin in Chinese) has been widely used in traditional Chinese medicine for treating hypertension (15), and inflammation (16). Over 30 flavonoids can be found in its dried root. Among these, Baicalein and Wogonin are two of the main active ingredients (17). Previous studies also demonstrated that Scutellaria baicalensis Georgi is a potent antifibrotic agent in animal models of hepatic fibrosis (18, 19). However, the potential antifibrotic effect of these flavonoids in the cardiovascular system has not been explored. In this study, collagen formation was induced by AngII in neonatal cardiac fibroblast from spontaneously hypertensive rats (SHR) and normotensive Wistar-Kyoto rats (WKY), which was used to investigate anti-fibrosis effect of baiklein and wogonin in the cardiac fibrosis.

*Corresponding author. Tel: 852-2632-3594; Fax: 852-2637-5643; E-mail: cmyu@cuhk.edu.hk

Received 3 February 2010, Accepted 21 February 2010

Keywords: Baicalein, Cardiac fibroblast, Collagen, Fibrosis, Wogonin
RESULTS AND DISCUSSION

Baicalein and Wogonin treatment attenuated COL-I and COL-III expression induced by AngII activation

AngII stimulation (100 nM concentration mimicking the condition in an ischemic heart, 20) in neonatal cardiac fibroblasts resulted in up-regulation of collagen production (both RNA and protein level, Table 1 and Fig. 1).

Renin-angiotensin system (RAS) had been studied as one of the determining factors in hypertension and its related diseases. Effect of AngII is exclusively controlled by the stimulation of AT1 receptors (21). AT2 receptor stimulation suggested a counter effect on AT1 (22). However, expression of AT2 receptor protein is not detectable in neonatal cardiac fibroblasts (23). AngII stimulation results in upregulation of collagen production without suppression (24) both in cultures from SHR and WKY, which provides a good in-vitro model for the current study.

With increasing dosage of Baicalein and Wogonin, there was progressive suppression of collagen production in neonatal cardiac fibroblasts activated by AngII (both RNA and protein level, Table 1 and Fig. 1). The COL-I and COL-III mRNA production in cardiac fibroblasts was suppressed by Baicalein (SHR on COL-I: 72.85 ± 8.96%, P < 0.01 and COL-III: 65.61 ± 14.17%, P < 0.01; WKY on COL-I: 77.47 ± 4.61%, P < 0.01; and COL-III: 79.17 ± 3.55%, P < 0.05) and high-dose Wogonin (SHR on COL-I: 67.69 ± 5.42%, P < 0.01 and COL-III: 82.40 ± 16.89%, P < 0.05; WKY on COL-I: 96.34 ± 9.54%, P < 0.05; and COL-III: 99.6 ± 10.00%), with an optimal dosage of 30 μM (Table 1).

Collagen protein expression was also assayed by Sirius red staining. Addition of either Baicalein or Wogonin significantly suppressed the total collagen expression in cardiac fibroblasts in higher concentration (30 μM (Fig. 1). When compared to normal control, Baicalein and Wogonin normalized the total collagen protein production in the presence of AngII, with an optimal dose of 30 μM (Fig. 1).

This is the first study of the kind that demonstrated the anti-fibrotic effect of Baicalein and Wogonin on cardiac fibroblasts. Both the total collagen level and the mRNA levels of COL-I and COL-III in the AngII activated cardiac fibroblasts were suppressed in a dose-dependent manner after incubation.

Table 1. Mean difference (%) of collagen mRNA (COL-I and COL-III) in CF between Baicalein and Wogonin treatment groups when compared with its corresponding AngII activated group

<table>
<thead>
<tr>
<th></th>
<th>Mean difference of collagen I mRNA</th>
<th>Mean difference of collagen III mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SHR</td>
<td>WKY</td>
</tr>
<tr>
<td></td>
<td>SHR</td>
<td>WKY</td>
</tr>
<tr>
<td>Baicalein (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AngII + 0.1 μM</td>
<td>−27.01 ± 14.02</td>
<td>−11.48 ± 8.70</td>
</tr>
<tr>
<td>AngII + 1 μM</td>
<td>−50.58 ± 11.85*</td>
<td>−16.80 ± 8.67</td>
</tr>
<tr>
<td>AngII + 10 μM</td>
<td>−40.12 ± 13.34</td>
<td>−40.64 ± 8.02*</td>
</tr>
<tr>
<td>AngII + 30 μM</td>
<td>−64.62 ± 10.30**</td>
<td>−60.02 ± 6.99**</td>
</tr>
<tr>
<td>Wogonin (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AngII + 0.1 μM</td>
<td>−14.34 ± 8.72</td>
<td>−24.07 ± 19.30</td>
</tr>
<tr>
<td>AngII + 1 μM</td>
<td>−5.64 ± 8.96</td>
<td>−30.51 ± 9.17</td>
</tr>
<tr>
<td>AngII + 10 μM</td>
<td>−30.64 ± 9.30*</td>
<td>−23.61 ± 8.25</td>
</tr>
<tr>
<td>AngII + 30 μM</td>
<td>−69.79 ± 9.07**</td>
<td>−41.15 ± 9.07**</td>
</tr>
</tbody>
</table>

n = 6 for each group. Bai: Baicalein, Wog: Wogonin. *P < 0.05 compare to AngII group. **P < 0.01 compare to AngII group.
of these flavonoids for 24 h. The collagen mRNA and protein expressions were normalized with increasing the dosage of Baicalein and Wogonin, with an optimal anti-fibrotic effect at 30 μM.

**Anti-fibrotic effect of Baicalein and Wogonin is mediated through ERK and MMP-9 pathways**

Addition of AngII and treatment with flavonoids altered the expression of 12-LO, ERK, p-ERK and MMP-9 expression (Fig. 2). Comparing to AngII group, application of Baicalein and Wogonin decreased the 12-LO expression level by dosage (from 0.1 to 30 μM). Though there were no significant changes in p-ERK expression in low dosage treatment group, the p-ERK expression of high dosage (30 μM) baicalein group significantly decreased compared to AngII group and high dosage wogonin group also slightly decreased. In contrast, ERK total protein expression was significantly upregulated by baicalein and wogonin incubation in SHR cardiac fibroblasts. ERK activity represents a ratio of pERK/ERK. As a result, ERK activity was significantly down-regulated by both Baicalein and Wogonin treatment (30 μM). For MMP9, AngII treatment enhanced the expression of MMP9 in SHR cardiac fibroblast. Interestingly, this enhancement was significantly attenuated by both Baicalein and Wogonin treatment (from 0.1 to 30 μM).

Previous studies suggested that AngII activates ERK (25) which induces collagen production through regulating levels of TIMPs and MMPs (26). Besides, 12-LO also plays a role in collagen deposition by cardiac fibroblasts. Increase of 12-LO expression would elevate the level of 12-HETE which then increases ERK activity (27). The activity of ERK (p-ERK/ERK ratio) depends on its degree of phosphorylation. A smaller value of p-ERK/ERK represents less ERK being activated. As shown in Fig. 2, treatments with both flavonoids reduced the 12-LO and ERK activity at optimal dosage of 30 μM. Decreased ERK activity results in attenuation of collagen production. MMPs and their inhibitors, TIMPs, are involved in cardiac remodeling with collagen (28). Studies also showed that MMP-9 expression correlated positively with cardiac fibrosis in Npr1 gene-disrupted mice (29). MMP-9 level was selectively up-regulated in congestive heart failure (30). However, the mechanism involving TIMPs and MMPs is still not entirely clear. MMP-9 could be activated by AngII through the activation of NF-κB pathway (31). In our study, MMP-9 level in cardiac fibroblast culture was up-regulated after AngII treatment. Both Baicalein and Wogonin attenuated the MMP-9 expression. It is likely that Baicalein and Wogonin, probably acting as a 12-LO inhibitor, inhibit the MMP-9 production through inhibition of ERK activation.

**Baicalein and Wogonin treatments have no cytotoxicity effect on cardiac fibroblasts**

AngII-activated SHR and WKY cardiac fibroblast were cultured with concentration of Baicalein and Wogonin ranging from 0.1 μM to 30 μM for 24 h. The cell viability and toxicity of com-

![Fig. 2](http://bmbreports.org)

Fig. 2. Western blots showing the effect of Baicalein and Wogonin on protein expression of 12-LO (A); ERK, p-ERK and p-ERK/ERK ratio (B); and MMP-9 (C) in SHR and WKY neonatal cardiac fibroblasts. Con: control, AngII: angiotensin II, Val: valsartan. n = 6 for each group. *P < 0.05 control vs. AngII group; **P < 0.05, ***P < 0.01 vs. AngII group.
pounds were evaluated by MTT and LDH efflux assay. The colorimetric reaction secondary to succinate dehydrogenase activity reflects the cell viability and cell membrane integrity. Comparing to AngII control group, the change of level of mitochondrial dehydrogenase and efflux of lactate dehydrogenase showed no significant changes among groups (Fig. 3A, B), which indicated that, both compounds showed no evidence of cytotoxicity even at optimal dosage of 30 μM.

In conclusion, this in-vitro study has demonstrated for the first time the antifibrotic effect of Baicalein and Wogonin, the two flavonoids extracted from Scutellaria baicalensis Georgi (Huang Qin in Chinese) in cardiac fibroblast cultures from SHR and WKY after activation by AngII. At an optimal dose of Baicalein and Wogonin of 30 μM, both suppressed pro-collagen I and III mRNA and protein production through suppressing 12-LO activity thus decreasing ERK activity. Another possible mechanism of action is through the normalization of MMP-9 level. Our results indicate the potential antifibrotic effect of Baicalein and Wogonin on the myocardium which suggests a potential therapeutic role for these flavonoids in the suppression of pathological myocardial fibrosis in hypertension.

MATERIALS AND METHODS

Experimental animals and ethic approval

Neonatal (1 to 4 days old) male spontaneously hypertensive rats (SHR) and normotensive Wistar-Kyoto rats (WKY) were obtained from Laboratory Animal Services Centre of The Chinese University of Hong Kong, Hong Kong, China. The handling of rats and all procedures performed were according to Animals (Control of Experiments) Ordinance (Cap.340), Hong Kong, China. Animal ethic approval was obtained from the Animal Ethic Committee of The Chinese University of Hong Kong (Ref. CUHK4318/04M).

Isolation of cardiac fibroblasts

Primary cultures were setup using a modified procedure reported by Fullerton and coworkers (32). In brief, rats were sacrificed and left ventricular apices of heart (n ≥ 6 for each group) were finely minced and placed together in 0.1% collagenase/2% BSA in DMEM at 37°C for 5 min with continuously shaking. They were then incubated with 0.05% collagenase/1% BSA in DMEM at 37°C for 35 min. Supernatant was combined in a tube containing 1 ml of FBS and temporary kept on ice. Digestion process was repeated 4 more times. The isolated single cells were centrifuged at 400 xg at 20°C for 10 min. Cells pellet was triturated in DMEM containing 10% FBS and antibiotics and was subsequently pre-plated at 37°C for 2 hr in the presence of 95% O2 /5% CO2. Attached fibroblasts were allowed to grow to confluence in the presence of 10% FBS/DMEM, non-essential amino acids, glutamine and antibiotics. In splitting ratio of 1:3, cells from passage 4 to 7 with 80 to 90% confluence were used. This isolation protocol established cardiac fibroblast cultures with purity ≥86% as validated by analysis of cardiac fibroblast-specific discoidin domain receptor 2 FACS (33).

Flavonoids incubation

Cardiac fibroblast cultures were pre-incubated in serum free DMEM for 24 hr. 100 nM AngII (Sigma, St. Louis, MO., USA)
was then added to cultures and treated for 24 hr by complete medium with non-essential amino acids and 0.15 mM L-ascorbic acid (Sigma, St. Louis, MO., USA). In studying the effect of flavonoids on collagen production of cardiac fibroblast cultures, Serial dilution (0.1, 1, 10 and 30 μM) of Baicalein (purity ≥ 83.75%) or Wogonin (purity ≥ 99.49%) (Purchased from Yunnan Yunyao Laboratory Company Limited, PR China) were co-incubated with AngII (100 nM). 10 μM Valsartan (generously gift from NOVARTIS Pharmaceuticals) was applied as control.

RNA isolation and quantitative real-time RT-PCR
Total RNA was isolated using Trizol Reagent according to the manufacturer's instructions (Invitrogen Life Technologies). Individual samples were subjected to DNase (Promega) treatment prior to qRT-PCR. Reverse transcription was performed by random hexamers (TagMan Reverse Transcription Reagents Kits, Applied Biosystems). Real-time PCR was performed on 1 ng of total RNA per reaction with TagMan Universal PCR Master Mix (Applied Biosystems). Primer sets (34, 35) were as follows: COL-I forward primer 5'-GCG AAG GCA ACA GTC GAT TC-3'; reverse primer 5'-CCC AAG TCG CGC GAC TC-3'; and TagMan probe 5'-6FAM-ACA GCA CGCT TGT GGA TGG CTG C-TAMRA-3'; COL-III forward primer 5'-CAG GAA TGG CTG C-TAMRA-3'; and TaqMan probe 5'-6FAM-ACA GCA CGCT TGT GGA TGG CTG C-TAMRA-3'. Normalization experiments were performed simultaneously for each sample with rodent 18S rRNA (Eukaryotic 18S rRNA Endogenous Control Kit, Applied Biosystems). The data obtained were calibrated with the endogenous control in the respective sample to obtain the relative expression levels. The relative expression level was calculated by $2^{-\Delta\Delta C_T}$ method (36).

Collagen protein quantification - Sirius Red assay
Sirius Red assay is specific for collagen staining which allows quantitative analysis (37). The total collagen protein in the myocardium was quantified by a method described by Junquiera and coworkers (38). In brief, cells were fixed by Bouin’s Fixative (5% (v/v) glacial acetic acid, 10% (v/v) aqueous formaldehyde in saturated aqueous picric acid) for 1 h at room temperature. Collagens were stained by 0.5% Sirius Red F3BA in saturated picric acid at room temperature for 1 h. The unbound dye was washed away by dipping quickly in running tap water. For complete dye elution, 250 μl of pre-warmed 0.1 N NaOH was added to each well, the plate was incubated at 37°C for 30 min with intermittent shaking. Eluted dye was quantified by absorbance at 540 nm using a microplate reader (SPECTRAmax Gemini, Bio-Tek Instruments, Inc.).

Western blotting
After 24 hr-flavonoid incubation, cells were lysed. Proteins were separated by SDS-PAGE and immunodetection (39) was performed with 12-LO, ERK, p-ERK, MMP, and GADPH primary antibodies (Santa Cruz Biotechnology Inc.) and HRP-conjugated secondary antibodies (Santa Cruz Biotechnology Inc.). The reactive bands were quantified by densitometry of radioautograph films (GS-700 imaging densitometer and Quantity One 1-D Analysis Software, Bio-Rad, Hercules, CA).

MTT and LDH efflux assay
Cells were seeded in 24-well plates. 24 hr after flavonoids treatments, cells were subjected for MTT assays. Toxicity was measured by Lactic Dehydrogenase (LDH) based in-vitro Toxicology Assay Kit (Sigma, St. Louis, MO., USA). Cytotoxicity was expressed as percentage release of LDH (LDH in culture medium/LDH in culture medium and cell lysate ×100%). The absorbance was read on a microplate reader at a wavelength of 570 nm with a background reading at 650 nm subtracted for MTT, at 490 nm with a background reading at 690 nm subtracted for LDH. Six readings for each group were averaged.

Statistical analysis
All results are expressed as mean ± SEM. Quantitative data underwent KS normal distribution test. Statistical analysis was performed by SPSS 13.0 for Windows (SPSS Inc., Illinois). If a significant interaction of treatment was documented (P value <0.05), values at single time points were compared by one-way ANOVA and Scheffe’s post-hoc test. A P value less than 0.05 was taken to indicate significant differences.

Acknowledgements
We gratefully acknowledge the support from the Research Grants Council of Hong Kong, China (RGC Reference number: 4318/04M). Valsartan was generously provided by NOVARTIS Pharmaceuticals.

REFERENCES


