Baicalein Attenuates Angiotensin II-Induced Cardiac Remodeling via Inhibition of AKT/mTOR, ERK1/2, NF-κB, and Calcineurin Signaling Pathways in Mice

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BACKGROUND
Baicalein, a specific lipoxygenase (LOX) inhibitor, has anti-inflammatory and antioxidant effects. However, the functional role of baicalein in angiotensin II (Ang II)-induced hypertension and cardiac remodeling remains unclear. Here we investigated the effect of baicalein on cardiac hypertrophy and fibrosis and the underlying mechanism.

METHODS
Wild-type (WT) mice were injected with Ang II (1,200 ng/kg/min) alone or together with 12/15-LOX inhibitor baicalein (25 mg/kg) for 14 days. Histological examinations were performed on heart sections with hematoxylin and eosin, Masson’s trichrome, wheat germ agglutinin staining, and immunohistochemistry. The messenger RNA (mRNA) expression of cytokines and protein levels were detected by real-time polymerase chain reaction (PCR) and western blot analysis respectively.

RESULTS
Ang II infusion significantly increased blood pressure but decreased cardiac contractile function reflected by fractional shortening% and ejection fraction% compared with saline-treated mice. Moreover, Ang II infusion resulted in marked cardiac hypertrophy and fibrosis, promoted accumulation of macrophages and T cells, the expression of proinflammatory cytokines and malondialdehyde (MDA) production. However, these actions were markedly reversed by administration of baicalein in mice. Mechanistically, the protective effects of baicalein were associated with the inhibition of inflammation, oxidative stress, and multiple signaling pathways (AKT/mTOR, ERK1/2, nuclear factor-κB (NF-κB), and calcineurin) in the Ang II-treated mice.

CONCLUSIONS
This study demonstrates that baicalein can significantly ameliorate Ang II-induced hypertension and cardiac remodeling, and may be a novel therapeutic drug for prevention of hypertensive heart diseases.

Keywords: 12/15-lipoxygenase; angiotensin II; baicalein; blood pressure; cardiac remodeling; hypertension; signaling pathways.

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Hypertension is one of the main risk factors for the development of cardiac remodeling leading to heart failure and death worldwide.¹,² Hypertensive cardiac remodeling consists of left ventricular (LV) hypertrophy, inflammation, and cardiac fibrosis.¹,² Previous studies have demonstrated that several mechanisms involved in the initiation and development of cardiac remodeling, among them, activation of the renin–angiotensin system plays a critical role in this process.² Angiotensin II (Ang II) is an octapeptide hormone that acts as growth factor to regulate cardiovascular homeostasis through angiotensin type 1 or type 2 receptors.² It also can activate a number of signaling pathways to contribute to hypertension and cardiac injury including hypertrophy, inflammation, and fibrosis.³,⁴ It has been reported that Ang II increases lipoxygenase (LOX) activity in macrophage and porcine smooth muscle in vitro via the angiotensin type 1 receptor.⁵,⁶ Activation of LOX mediates Ang II-induced vascular actions and hypertension in mice and rats, but treatment with LOX inhibitors blocks these effects.⁷,⁸ These data suggest that LOX may play an important role in Ang II-induced cardiovascular remodeling.

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12/15-lipoxygenase (12/15-LOX) is a member of the LOX family that catalyzes the step from arachidonic acid to hydroxy-eicosatetraenoic acids, which is widely expressed in heart, blood vessels, and other organs. Accumulating evidence indicates that activation of 12/15-LOX have critical roles in the development of various cardiovascular diseases, such as atherosclerosis, myocardial ischemia/reperfusion (I/R) injury, heart failure, and hypertensive diseases. Thus, targeting 12/15-LOX will hopefully provide a promising therapeutic strategy for treatment of these diseases. Baicalein, a traditional Chinese medicine derived from the root of Scutellaria baicalensis Georgi, is a specific LOX inhibitor that exhibits antioxidant activity and anti-inflammatory effects. Recently, our and other studies have demonstrated that baicalein exerts protective effects against I/R injury, hypertension and cardiac dysfunction in mouse or rat models. However, the effect of baicalein on Ang II-induced cardiac remodeling remains unclear.

It has been known that Ang II can induce cardiac remodeling through both Ang II-mediated signaling and blood pressure elevation, therefore, here we aimed to test the effect of baicalein on hypertension and Ang II-induced inflammation and oxidative stress as well as several signaling pathways. Our results demonstrated that inhibition of 12/15-LOX with baicalein significantly attenuated Ang II-induced elevation of blood pressure, cardiac hypertrophy, and fibrosis. These beneficial effects were associated with inhibition of inflammation, oxidative stress, and multiple signaling pathways (AKT/mTOR, ERK1/2, nuclear factor-κB (NF-κB), and calcineurin). These results support that baicalein, a 12/15-LOX-specific inhibitor, could be a promising new therapeutic strategy for treatment of these diseases.

METHODS

Antibodies and reagents

The antibodies against Mac-2 and CD3 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-AKT, phospho-AKT (Ser473), mTOR, phospho-mTOR (Ser2448), ERK1/2, phospho-ERK1/2 (Thr202/Tyr204), calcineurin, NF-κB/p65, phospho-NF-κB/p65 (Ser536), PTEN, GAPDH, and horseradish peroxidase-linked anti-mouse or rabbit IgG antibody were obtained from Cell Signaling Technology (Danvers, MA). Baicalein and TRITC-labeled wheat germ agglutinin were from Santa Cruz Biotechnology. Ang II was from Sigma, Inc. (Sigma-Aldrich, St. Louis, MO). Penicillin, streptomycin, and fetal bovine serum were obtained from Invitrogen Life Technologies (Carlsbad, CA). Other reagents were purchased from Sigma-Aldrich or Invitrogen Life Technologies.

Animals and treatment

Male wild-type (WT) mice (C57BL/6, 10- to 12-week-old) were implanted with osmotic mini-pumps (Alzet, Cupertino, CA) subcutaneously and infused with vehicle (saline) or Ang II at 1,200 ng/kg/min in Ringer’s solution (0.01 mmol/l acetic acid in saline) for 14 days. A well-established mouse model of cardiac hypertrophy and remodeling induced by Ang II was performed as described. In briefly, 50–60 μg protein was loaded on sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and the proteins were transferred to nitrocellulose membranes. The membranes were then incubated with appropriate primary antibodies, followed by incubation with the corresponding secondary antibodies conjugated with horseradish peroxidase. The bands were visualized using enhanced chemiluminescence. The transcript levels of atrial natriuretic factor (ANF), β-myosin heavy chain (β-MHC), interleukin-6, interleukin-1β, tumor necrosis factor-α, collagen I, collagen III, and α-smooth muscle actin (α-SMA) were determined by quantitative real-time PCR analysis. Digital photographs were taken at ×200 magnification of over 20 random fields from each heart, and the positive areas were analyzed by Image Pro Plus 3.0 (ECLIPSE 80i/90i; Nikon, Tokyo, Japan).

Histological analysis

After saline or Ang II infusion, hearts were fixed in 4% paraformaldehyde solution, embedded in paraffin and sectioned (5 μm). Heart sections were stained with hematoxylin and eosin and Masson’s trichrome using standard procedure. Sections were also stained with 50 μg/ml of TRITC-labeled wheat germ agglutinin for 60 min to evaluate cardiomyocyte cross-sectional area. The cell areas were calculated by measuring at least 200 cells per slide. Immunohistochemistry was performed with primary antibodies, including Mac-2 (1:200) and CD3 (1:200). Digital photographs were taken at ×200 magnification of over 20 random fields from each heart, and the positive areas were analyzed by Image Pro Plus 3.0 (ECLIPSE 80i/90i; Nikon, Tokyo, Japan).

Quantitative real-time PCR analysis

Total RNA was isolated from fresh mouse hearts by using Trizol method according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA). The transcript levels of atrial natriuretic factor (ANF), β-myosin heavy chain (β-MHC), interleukin-6, interleukin-1β, tumor necrosis factor-α, collagen I, collagen III, and α-smooth muscle actin (α-SMA) were determined by quantitative real-time polymerase chain reaction (PCR) analysis. Western blot analysis

Proteins were prepared from heart tissues, and western blot analysis was performed as described. In briefly, 50–60 μg protein was loaded on sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and the proteins were transferred to nitrocellulose membranes. The membranes were then incubated with appropriate primary antibodies, followed by incubation with the corresponding secondary antibodies conjugated with horseradish peroxidase. The bands were visualized using enhanced chemiluminescence.
gel electrophoresis gels, transferred to polyvinylidene difluoride membranes, and incubated with primary antibodies against AKT, phospho-AKT, mTOR, phospho-mTOR, ERK1/2, phospho-ERK, calcineurin, NF-κB/p65, phospho-NF-κB/p65, PTEN (all at a dilution of 1:800–1,000), and glyceraldehyde 3-phosphate dehydrogenase (1:3,000), then with horseradish peroxidase-conjugated secondary antibodies (1:2,500). All blots were developed by using a chemiluminescent system, and densitometry analysis involved a Gel-pro 4.5 Analyzer (Media Cybernetics, Bethesda, MD).

Measurement of MDA levels

The levels of malondialdehyde (MDA) in the heart tissues of Ang II-treated mice with or without baicalein were examined with colorimetric assay kit according to the manufacturer’s instruction as previously described.

Statistical analysis
 Data are expressed as mean ± SEM. Statistical analyses involved use of one-way analysis of variance followed by t-test for multiple comparisons within treatment groups with use of SPSS 13.0 (SPSS Inc., Chicago, IL). P < 0.05 was considered statistically significant.

RESULTS

Ang II infusion increases 12/15-LOX expression and baicalein attenuates this effect

To investigate the role of 12/15-LOX in Ang II-induced cardiac remodeling, we first detected the expression of 12/15-LOX in WT mice. Immunohistochemical staining showed that Ang II infusion for 14 days significantly increased 12/15-LOX expression as compared with saline (Supplementary Figure 1a). Western blot analysis further confirmed these results (Supplementary Figure 1b). In contrast, baicalein treatment markedly attenuated this effect (Supplementary Figure 1a,b). These results suggest that Ang II treatment can induce myocardial 12/15-LOX expression.

Baicalein attenuates Ang II-induced hypertension and improves cardiac contractile function

To investigate the effect of baicalein on Ang II-induced hypertension and cardiac function, WT mice were infused with Ang II (1,200 ng/min/kg) in the presence or absence of baicalein (25 mg/kg) for 14 days. Ang II infusion markedly increased systolic blood pressure compared with saline-treated mice, and this effect was markedly attenuated in baicalein-treated mice (Figure 1a). No difference in the blood pressure was observed between two groups after saline infusion (Figure 1a). Moreover, Ang II infusion also caused vascular hypertrophy, thickening, and breaks of the elastic fibers in WT mice, and these histological alterations were significantly blunted in baicalein-treated mice (Supplementary Figure 2), indicating that baicalein decreases Ang II-induced hypertension partially by inhibiting vascular remodeling.

Echocardiography revealed that LV internal dimension at diastole and LV volume at diastole in Ang II-treated mice were higher than that in saline-treated mice (Figure 1c,d). The mean values of %LV ejection fraction and %LV fractional shortening in Ang II-treated mice were markedly decreased as compared to saline-treated animals (Figure 1e,f). In contrast, administration of baicalein significantly reversed these effects (Figure 1c–f). There was no difference in all echocardiographic parameters (including LV internal dimension at diastole, LV volume at diastole, %LV ejection fraction, and %LV fractional shortening) between two groups after saline infusion (Figure 1c–f). Thus, these results suggest baicalein can ameliorate Ang II-induced hypertension and cardiac dysfunction in mice.

Baicalein inhibits Ang II-induced cardiac hypertrophy and fibrosis

To evaluate the effect of baicalein on cardiac hypertrophy after Ang II infusion, we measured the LV mass to body weight (LV mass/body weight) ratio and examined heart sections with wheat germ agglutinin staining. Consistent with echocardiographic measurements, Ang II infusion significantly increased the LV mass/body weight ratio and cross-sectional area of cardiomyocytes in LV tissues of WT mice, but this effect was largely inhibited in baicalein-treated hearts (Figure 2a,b). Moreover, quantitative PCR analysis confirmed that the messenger RNA (mRNA) expression of hypertrophic markers, including ANF and β-MHC, was also markedly lower in baicalein-treated mice than that in control group after Ang II infusion (Figure 2c). There was no difference in LV mass/body weight ratio, cardiomyocyte cross-sectional areas and hypertrophic markers between two groups after saline infusion (Figure 2a–c). Together, these results suggest that baicalein can suppress Ang II-induced cardiac hypertrophy.

To determine whether baicalein reduces Ang II-induced cardiac fibrosis, we examined collagen deposition and expression in the hearts by Masson's trichrome staining. As shown in Figure 2d,e, Ang II infusion significantly increased cardiac fibrotic areas, the mRNA levels of collagen I and collagen III (fibrotic markers) as well as α-SMA (a myofibroblast marker) compared with saline-treated mice, whereas administration of baicalein markedly attenuated these effects. Collectively, these results indicate that baicalein can inhibit Ang II-induced cardiac hypertrophy and fibrosis.

Baicalein reduces Ang II-induced myocardial inflammation and oxidative stress

Since baicalein has anti-inflammatory and antioxidant effects, we then assess whether baicalein affects myocardial inflammation and oxidative stress induced by Ang II. Histological examinations showed that Ang II infusion significantly increased the numbers of inflammatory cells including Mac-2-positive macrophages and CD3-positive lymphocytes in the heart tissues as compared with saline-treated group, whereas these effects were markedly attenuated in baicalein-treated hearts.
Baicalein Inhibits Angiotsin II-Induced Cardiac Remodeling

Furthermore, quantitative PCR analysis confirmed that the expression of proinflammatory cytokines including interleukin-6, interleukin-1β, and tumor necrosis factor-α in baicalein-treated hearts were also significantly lower than that in untreated mice after Ang II infusion. In addition, MDA is considered as an indirect marker for oxidative stress, we therefore measured MDA levels in the hearts. Ang II infusion markedly increased cardiac MDA levels as compared with saline-treated groups. Conversely, administration of baicalein markedly attenuated this effect. Together, these data suggest that baicalein can reduce Ang II-induced inflammation and oxidative stress in the heart.

Figure 1. Baicalein reduces blood pressure elevation and enhances contractile function after angiotensin II (Ang II) infusion. (a) Mice were infused with Ang II (1,200 ng/min/kg) without or with baicalein treatment for 14 days. Noninvasive blood pressure was measured in mice. (b) Representative echocardiogram of the cardiac function of mice (M-mode, parasternal short axis) for each condition. (c,d) Quantification of left ventricular (LV) internal dimension at diastole (LVID; d) and LV volume at diastole (LV Vol; d). (e,f) Quantification of LV ejection fraction (% LVEF) and LV fractional shortening (% LVFS). Data are expressed as mean ± SEM (n = 7 per group). *P < 0.01 vs. saline; #P < 0.05 vs. Ang II-treated mice.
Figure 2. Baicalein inhibits angiotensin II (Ang II)-induced cardiac hypertrophy and fibrosis. (a) The ratio of left ventricular mass (LV mass)/body weight from saline and Ang II-treated mice. (b) WGA staining of cardiomyocyte size in the heart sections of saline and Ang II-treated mice (top). Quantification of cardiomyocyte cross-sectional areas (bottom). Bar: 50 μm. (c) Quantitative polymerase chain reaction (qPCR) analysis of the messenger RNA (mRNA) expressions of ANF and β-MHC in heart tissues treated with saline or Ang II in the presence or absence of baicalein. (d) Representative Masson’s trichrome-stained heart sections (top). Quantification of fibrotic areas (bottom). Bar: 100 μm. (e) qPCR analysis of the mRNA levels of collagen I, collagen III, and α-SMA in the heart tissues treated with saline or Ang II in the presence or absence of baicalein. Data are expressed as mean ± SEM (n = 6 per group). *P < 0.01 vs. saline; #P < 0.05 vs. Ang II-treated mice. Abbreviation: WGA, wheat germ agglutinin.
Baicalein Inhibits Angiotensin II-Induced Cardiac Remodeling

Baicalein inhibits activation of AKT/mTOR, ERK, NF-κB, and calcineurin signaling pathways stimulated by Ang II

To elucidate the molecular mechanism of baicalein to protect against Ang II-mediated hypertensive cardiac remodeling, we tested the activation of several signaling pathways including PTEN/AKT/mTOR, ERK, NF-κB, and calcineurin, which are well known to play critical roles in regulating hypertrophy, fibrosis, and inflammation by western blot analysis. Ang II infusion significantly increased the relative levels of PTEN, phosphorylated AKT, mTOR, ERK1/2, and NF-κB/p65 as well as calcineurin protein expression but did not affect PTEN protein level (Figure 4a–d, Supplementary Figure 3). There was no significant difference in the levels of these proteins between two groups after saline treatment (Figure 4a–d, Supplementary Figure 3).

To further determine which signaling pathway participates the protective effect of baicalein on Ang II-induced cardiac hypertrophy, cardiomyocytes were pretreated with the specific inhibitors, including wortmannin (PI3 kinase inhibitor), U0126 (MEK1/2 inhibitor), PDTC (NF-κB/p65 inhibitor), and cyclosporin A (calcineurin inhibitor). The surface area of cardiomyocytes and ANF expression (a marker of hypertrophy) were examined. As shown in Supplementary Figure 4a,b, Ang II treatment significantly

Figure 3. Baicalein decreases infiltration of inflammatory cells and proinflammatory cytokine expression and oxidative stress in the heart after angiotensin II (Ang II) infusion. (a) Hematoxylin and eosin (H&E) staining (bar: 100 μm) and immunohistochemistry with antibodies against Mac-2, CD3 (bar: 50 μm) of heart sections treated with saline or Ang II (top). Quantification of Mac-2- and CD3-positive cells (bottom). (b) Quantitative polymerase chain reaction analysis of the messenger RNA (mRNA) levels of interleukin (IL)-6, IL-1β, and tumor necrosis factor-α (TNF-α) in heart tissues. (c) MDA levels were measured in heart homogenates from saline and Ang II-treated mice without and with baicalein. Data are expressed as mean ± SEM (n = 6 per group). *P < 0.01 vs. saline; #P < 0.05 vs. Ang II-treated mice. Abbreviation: MDA, malondialdehyde.
increased the surface areas of cardiomyocytes and the expression levels of ANF mRNA compared with control (compared lanes 2 with 1), whereas baicalein treatment markedly attenuated these effects (compared lanes 3 with 2). Moreover, treatment of cells with wortmannin, U0126, PDTC, or cyclosporin A alone significantly attenuated Ang II-induced increase of surface areas of cardiomyocytes and ANF expression (compared lanes 4, 6, 8, and 10 with 2). Furthermore, combined treatment with baicalein and wortmannin, U0126, PDTC, or cyclosporin A suppressed the effects of Ang II-induced increase of surface areas and ANF mRNA levels (compared lanes 5, 7, 9, and 11 with 3) (Supplementary Figure 4a,b). Taken together, these results suggest that baicalein may protect against Ang II-induced...
cardiac hypertrophy via modulation of AKT/mTOR, ERK, NF-kB, and calcineurin signaling pathways.

**DISCUSSION**

Baicalein, a specific inhibitor for 12/15-LOX, has protective role in ameliorating hypertension, I/R injury, and other diseases in animals. However, little is known about the molecular mechanism of baicalein on Ang II-induced cardiac remodeling. In this study, we demonstrated that Ang II infusion significantly induced hypertension, cardiac hypertrophy, fibrosis and caused cardiac contractile function, whereas administration of baicalein markedly attenuated these effects. Mechanistically, these beneficial actions were associated with baicalein-mediated inhibition of inflammation, oxidative stress, and multiple signaling pathways in Ang II-treated hearts.

Cardiac hypertrophy is a major risk factor associated with heart failure. There are two structural changes that occur in the hypertrophied heart during hypertension: cardiomyocyte hypertrophy and cardiac fibroblast growth. Cardiac fibrosis is a structural determinant of LV stiffness and diastolic dysfunction. Several studies demonstrate that overexpression of 12/15-LOX enhances Ang II-induced cardiac fibroblast cell growth and hypertrophy, indicating that 12/15-LOX mediates interactions of Ang II with the cardiac fibroblasts and myocytes leading to growth, and increases protein synthesis and extracellular matrix gene expression. Interestingly, administration of baicalein for 12 weeks significantly reduced the LV procollagen expression, systolic and diastolic intraventricular septum thickness in spontaneously hypertensive rats. Our results further confirmed that baicalein treatment markedly attenuated Ang II-induced cardiac hypertrophy and fibrosis, improved contractile function in mice (Figures 1 and 2).

Inflammation and oxidative stress play critical roles in regulating cardiac remodeling and function. Many studies show that baicalein has anti-inflammatory, antioxidative stress activities in various inflammatory and other diseases. Baicalein can suppress inflammation by inhibiting NF-kB/p65 activation in cigarette smoke-induced model of chronic obstructive pulmonary disease. Baicalein also improves cardiac contractility in lipopolysaccharide-induced sepsis by the inhibition of inflammatory cytokines and cardiomyocyte apoptosis. Furthermore, baicalein treatment protects I/R-induced cardiomyocyte apoptosis by inhibiting oxidant stress. In this study, our data indicated that baicalein treatment markedly suppressed Ang II-induced inflammation and MDA production (Figure 3a–c), which was accompanied by inhibition of NF-kB/p65 signaling pathway (Figure 4c). Together, these findings suggest that administration of baicalein exerts anti-inflammatory and antioxidant effects in Ang II-infused mice. Further investigations in other Ang II models are required to determine the clinical application of baicalein as a novel drug.

It has been reported that Ang II can induce cardiac remodeling through blood pressure elevation and angiotensin type I receptor-mediated inflammation and oxidative stress. Baicalein has been known to lower blood pressure in renin-dependent hypertension or spontaneously hypertensive rats via inhibition of production of endothelial nitric oxide and inducible nitric oxide synthase (iNOS) gene expression. Consistent with these findings, our results also demonstrated that administration of baicalein decreased Ang II-induced hypertension and vessel injury (Supplementary Figure 2) in mice (Figure 1). Increasing evidence suggests that accumulation of proinflammatory cells, including monocytes/macrophages, T cells but not B cells in mouse vessel contributes to Ang II-induced hypertension. Moreover, the present results showed that baicalein treatment significantly inhibited cardiac inflammation and MDA production (Figure 3). However, whether baicalein exerts similar anti-inflammatory and antioxidant effects in vascular tissue remains to be explored.

Emerging evidence demonstrates that 12/15-LOX plays an important role in regulating cell growth, apoptosis, and function of endothelial cells, smooth muscle cells, macrophages, cardiac fibroblasts, cardiomyocytes, and other cell types. 12/15-LOX regulates them through multiple signaling pathways including PI3K/AKT, MAPks, STAT3, and NF-kB/p65, which have central roles in controlling cardiac hypertrophy, apoptosis, fibrosis, inflammation, and reactive oxygen species production. Our results showed that administration of baicalein significantly attenuated Ang II-induced expression of phosphorylated AKT/mTOR, ERK1/2, NF-kB/p65, and calcineurin in mouse hearts (Figure 4), indicating that baicalein can effectively inhibit Ang II-induced cardiac remodeling at least in part through the inhibition of these signaling pathways.

In conclusion, the present results provide novel evidence for the functional role of baicalein in preventing Ang II-induced hypertension and cardiac damage. Baicalein not only inhibited cardiomyocyte growth and collagen deposition but also reduced inflammation, subsequent cardiac remodeling and dysfunction in Ang II-induced hypertensive hearts. The protective actions of baicalein were associated with inhibition of Ang II-induced cardiac inflammation, oxidative stress, and multiple signaling pathways. These findings suggest that baicalein may be a new potential drug for treatment of hypertensive heart disease.

**SUPPLEMENTARY MATERIAL**

Supplementary materials are available at American Journal of Hypertension (http://ajh.oxfordjournals.org).

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**DISCLOSURE**

The authors declared no conflict of interest.
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