Original article

Inhibitory effect of alpinate Oxyphyllae fructus extracts on Ang II-induced cardiac pathological remodeling-related pathways in H9c2 cardiomyoblast cells

Yung-Ming Chang\textsuperscript{a,b}, Bharath Kumar Velmurugan\textsuperscript{c}, Wei-Wen Kuo\textsuperscript{d}, Yueh-Sheng Chen\textsuperscript{a}, Tsung-Jung Ho\textsuperscript{a,e}, Chuan-Te Tsai\textsuperscript{b}, Chi-Xin Ye\textsuperscript{c}, Chang-Hai Tsai\textsuperscript{f}, Fuu-Jen Tsai\textsuperscript{a,g}, Chih-Yang Huang\textsuperscript{a,c,h,*}

\textsuperscript{a}School of Chinese Medicine, China Medical University, Taichung, Taiwan
\textsuperscript{b}1PT Biotechnology Co. Ltd., Taichung, Taiwan
\textsuperscript{c}Graduate Institute of Basic Medical Science, China Medical University, Taichung, Taiwan
\textsuperscript{d}Department of Biological Science and Technology, China Medical University, Taichung, Taiwan
\textsuperscript{e}Chinese Medicine Department, China Medical University Beigang Hospital, Beigang, Taiwan
\textsuperscript{f}Department of Healthcare Administration, Asia University, Taichung, Taiwan
\textsuperscript{g}Department of Pediatrics, Medical Research and Medical Genetics, China Medical University, Taichung, Taiwan
\textsuperscript{h}Department of Health and Nutrition Biotechnology, Asia University, Taichung, Taiwan

\textbf{A B S T R A C T}

Background: Our previous studies have demonstrated that Ang II induced IGF-II and IGF-II R via ERK and JNK signaling pathways and further induces cardiac cell apoptosis.

Purpose: The present study investigates the protective role of alpinate Oxyphyllae fructus (AOF;\textit{Alpinia oxyphylla} Miq) extracts on angiotensin II (Ang II)-stimulated H9c2 cardiomyoblast cells.

Methods: Western blotting was used to analyze the molecular mechanism involved in Ang II-treated H9c2 cells.

Results: AOF inhibits cardiac hypertrophy, apoptosis, mitochondrial dysfunction, and cardiac remodeling in Ang II-treated H9c2 cells.

Conclusion: All these data collectively suggest us that, AOF significantly inhibits Ang-II induced H9c2 cells apoptosis by suppressing the mitochondrial apoptotic pathway.

*Corresponding author. School of Chinese Medicine and Graduate Institute of Basic Medical Science, China Medical University, Number 91, Hsueh-Shih Road, Taichung 404, Taiwan.
E-mail address: cyhuang@mail.cmu.edu.tw (C.-Y. Huang).
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1. Introduction

With a constant increase in life expectancy, the elderly population is expanding rapidly (conventionally, a cutoff point for advanced age in humans may be set at 65 years). The magnitude of the problem arising from this factor was emphasized in a recent report stating that individuals normotensive at 55 years of age have a 90% lifetime risk of developing hypertension [1]. Therefore, aging is often considered a significant risk for cardiac disease [2]. In the elderly, systolic blood pressure increases because of the arterial stiffness produced by structural alterations of arterial walls that occur with aging [3]. Consequently, aging poses major health concerns, and quite commonly contributes to cardiovascular morbidity and mortality via severe heart damage.

Angiotensin II (Ang II) plays an important role in cardiovascular diseases, for example, hypertension, atherosclerosis, left ventricular hypertrophy (LVH), and heart failure [4–10]. Most studies demonstrate that Ang II induces cardiovascular hypertrophy, cardiac apoptosis, mitochondrial dysfunction, and cardiac remodeling through activation of the Ang II type 1 receptor (AT1R) [4,7,11,12]. However, the opposite theory indicates AT2R causes opposite effects, for example, cardiac growth-promoting effects [13–17]. Although the functions of both major Ang II receptors prove ambiguous, the harm caused by Ang II to cardiomyocytes is beyond question [10,18–22]. In cardiac hypertrophy, calcium-dependent phosphatase calcineurin dephosphorylates the nuclear factor of activated T cells 3 (NFAT3) transcription factor, allowing it to translocate to the nucleus, after which atrial natriuretic (ANF) and b-type natriuretic peptide (BNF) are overexpressed. Gq signaling and the mitochondrial membrane potential play vital roles in cardiomyocyte apoptosis [23].

Insulin-like growth factor II (IGF-II) also stimulates myocardial hypertrophy, apoptosis, and remodeling [24–27]. Our prior study demonstrated that Ang II seems to induce IGF-II and IGF-II R via extracellular signal-regulated kinases (ERK) and c-Jun N-terminal kinase (JNK) signaling pathways, respectively, and further activates cardiac cell apoptosis through calcineurin-dependent pathways [28], ultimately causing heart failure.

Alpinia oxyphylla fructus (AOF; Alpinata Oxyphyllae fructus; IGF) ranks among the most important traditional Chinese medicines and has been used to treat diarrhea, polyuria, ulceration, dementia, tumors, and gastralgia, according to Chinese Pharmacopoeia [29]. Several experiments indicate its potential as a neuroprotective agent, both in water and ethanol extracts [29–33]. In Korea, AOF serves as a medicinal plant, also used to treat various symptoms accompanying hypertension and cerebrovascular disorders [30].

Methanol extract of AOF reportedly has cardiotonic effectiveness [34]; whether it has protective and rescue effects on Ang II-stimulated H9c2 cardiomyocytes remains unknown. This study evaluates the pathophysiological mechanisms of AOF in cardiac hypertrophy, apoptosis, and the mitochondrial dysfunction and remodeling induced by Ang II treatment in cardiomyoblast H9c2 cells.

2. Materials and methods

2.1. AOF extraction

We purchased AOF in fragmented form from Shin-Long Pharmaceutical Company (Taichung, Taiwan); 150 g of AOF fragment was extracted with 600 mL of boiling water for 2 hours. The filtrate was concentrated at reduced pressure for convenience. The extract solution was stored at 4°C and spray-dried to yield a powdered extract.

2.2. Cell culture

H9c2 cardiomyoblasts from the American Type Culture Collection (ATCC, CRL-1446, Rockville, MD, USA) were cultured in 100-mm dishes in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 100 µg/mL penicillin, 100 µg/mL streptomycin, 2 mM glutamine, 1 mM HEPS buffer, and 10% fetal bovine serum in humidified air (5% CO2) at 37°C. H9c2 cells were incubated in serum-free essential medium for 4 hours before treatment with the indicated agents.

2.3. Immunoblotting

To isolate total proteins, cultured myocardiac cells were washed with cold phosphate-buffered saline (PBS) and resuspended in lysis buffer [50 mM Tris, pH 7.5, 0.5 M NaCl, 1.0 mM EDTA, pH 7.5, 10% glycerol, 1 mM B-Mercaptoethanol (BME), 1% octylphenyl-polyethylene glycol (IGEPAL-630), and proteinase inhibitor cocktail (Roche Molecular Biochemicals, IN, USA)]. After 30 minutes incubation on ice, the supernatant was collected by centrifugation at 12,000 rpm for 30 minutes at 4°C. The protein concentration was determined by the Bradford method. Samples with equal proteins (35 µg) were loaded and analyzed by Western blotting. Briefly, proteins were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Belfor, MA, USA). Membranes were

![Fig. 1](image-url) - The effect of AOF on Ang II-induced IGF-II/II R signaling in H9c2 cells. H9c2 cells at 80% confluence were treated with Ang II (5 x 10^{-7} M) for 1 hour, then administered AOF (20, 40, 60, 80, and 100 µg/mL) for a further 23 hours. The total protein of cell extracts was separated by 12% SDS-PAGE, transferred to PVDF membranes, and immunoblotted with antibodies against IGF-II and Gαq proteins. Equal loading was assessed with anti-β-actin antibody. Cell culture without treatment served as control. Ang II = angiotensin II; AOF = alpinata Oxyphyllae fructus; IGF = insulin-like growth factor II; PVDF = polyvinylidene fluoride; SDS-PAGE = sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
blocked with buffer (5% non-fat dry milk, 20 mM Tris-HCl, pH 7.6, 150 mM NaCl, and 0.1% Tween 20) for at least 1 hour at room temperature and incubated with primary antibodies in the above solution on an orbit shaker at 4°C overnight. Following primary antibody incubations, membranes were incubated with horseradish peroxidase-linked secondary antibodies [anti-rabbit, anti-mouse, or anti-goat immunoglobulin G (IgG)].

2.4. Statistical analysis

Each experiment was triplicated, and the results were presented as mean ± standard error of the mean (SEM). Statistical comparisons were performed using the Student t test, with \( p < 0.05 \), \( p < 0.001 \), or \( p < 0.0001 \) deemed significant.

3. Results

3.1. AOF down-regulates Ang II-induced IGF-II/II R signaling in H9c2 cells

A previous study demonstrated that Ang II seems to evoke IGF-II and IGF-II R and further activates cardiac cell apoptosis via G\( \alpha_q \) downstream effectors. To ascertain whether AOF inhibits Ang II-induced IGF-II and G\( \alpha_q \), these proteins were measured after AOF post-treatment in Ang II-treated H9c2 cells. Fig. 1 shows IGF-II and G\( \alpha_q \) increasing with Ang II stimulation and decreasing with AOF administration, which indicates that anti-IGF-II/II R signaling induced by Ang II arises during AOF administration.

3.2. AOF inhibits calcineurin-dependent apoptosis in Ang II-stimulated H9c2 cells

To clarify whether AOF would prevent Ang II-induced apoptosis in H9c2 cells, expression levels of calcineurin-dependent apoptosis proteins were measured. H9c2 cells at 80% confluence were treated with Ang II (5 \( \times \) 10\(^{-7} \) M) for 1 hour, then administered AOF (20, 40, 60, 80, and 100 \( \mu g/\)mL) for a further 23 hours. The higher levels of calcineurin and cytochrome c induced by Ang II fell after AOF treatment (Fig. 2), indicating that AOF inhibits the calcineurin-dependent apoptotic protein expression activated by Ang II in H9c2 cells.

3.3. AOF reduces Ang II-induced hypertrophic protein expression in Ang II-induced H9c2 cells

To clarify whether AOF would prevent Ang II-induced hypertrophy in H9c2 cells, expression levels of hypertrophic proteins BNP and ANP were measured. H9c2 cells at 80% confluence were treated with Ang II (5 \( \times \) 10\(^{-7} \) M) for 1 hour, then administered AOF (20, 40, 60, 80, and 100 \( \mu g/\)mL) for a further 23 hours. Higher BNP and ANP levels fell after AOF treatment (Fig. 3), indicating that AOF inhibits the hypertrophic BNP and ANP expression levels activated by Ang II in H9c2 cells.

3.4. AOF inhibits remodeling protein expression in Ang II-stimulated H9c2 cells

To clarify whether AOF would prevent Ang II-induced cardiac remodeling in H9c2 cells, the remodeling proteins were measured. Ang II-treated H9c2 cells showed increasing levels of specificity protein 1 (Sp1) and connective tissue growth factor (CTGF), which then decreased with AOF administration (Fig. 4). The results indicate that cardiac remodeling proteins were inhibited by AOF post-treatment of Ang II-treated H9c2 cells.

4. Discussion

This study proved that post-treatment of water extract AOF definitely reduced the cardiomyoblast cell pathological hypertrophy, apoptosis, and remodeling caused by angiotensin II (Ang II) challenge in H9c2 cells. AOF also inhibited the IGF-II/II R-related signaling pathway, including G\( \alpha_q \)- and calcineurin-dependent pathways. Our previous studies found that Ang II

![Image 1](https://example.com/image1)

**Fig. 2** – AOF down-regulated calcineurin and cytochrome c in Ang II-treated H9c2 cells. H9c2 cells at 80% confluence were treated with Ang II (5 \( \times \) 10\(^{-7} \) M) for 1 hour, then administered AOF (20, 40, 60, 80, and 100 \( \mu g/\)mL) for a further 23 hours. The total protein of cell extracts was separated by 12% SDS-PAGE, transferred to PVDF membranes, and immunoblotted with antibodies against calcineurin and cytochrome c proteins. Equal loading was assessed with anti-\( \beta \)-actin antibody. Cell culture without treatment served as control. Ang II = angiotensin II; AOF = alpinate Oxyphyllae fructus; PVDF = polyvinylidene fluoride; SDS-PAGE = sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

![Image 2](https://example.com/image2)

**Fig. 3** – The effect of AOF on Ang II-induced hypertrophy in H9c2 cells at 80% confluence treated with Ang II (5 \( \times \) 10\(^{-7} \) M) for 1 hour, then administered AOF (20, 40, 60, 80, and 100 \( \mu g/\)mL) for a further 23 hours. The total protein of cell extracts was separated by 12% SDS-PAGE, transferred to PVDF membranes, and immunoblotted with antibodies against BNP and ANP proteins. Equal loading was assessed with anti-\( \beta \)-actin antibody. Cell culture without treatment served as control. Ang II = angiotensin II; ANP = atrial natriuretic peptide; AOF = alpinate Oxyphyllae fructus; BNP = \( b \)-type natriuretic peptide; PVDF = polyvinylidene fluoride; SDS-PAGE = sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
Fig. 4 — AOF prevented Ang II-induced specificity protein 1 (Sp1) and connective tissue growth factor (CTGF) in Ang II-treated H9c2 cells. H9c2 cells at 80% confluence were treated with Ang II (5 × 10^{-7} M) for 1 hour, then administered AOF (20, 40, 60, 80, and 100 μg/mL) for a further 23 hours. The total protein of cell extracts was separated by 12% SDS-PAGE, transferred to PVDF membranes, and immunoblotted with antibodies against Sp1 and CTGF proteins. Equal loading was assessed with anti-β-actin antibody. Cell culture without treatment served as control. Ang II = angiotensin II; AOF = alpine Oxyphyllae fructus; CTGF = connective tissue growth factor; PVDF = polyvinylidene fluoride; SDS-PAGE = sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Sp1 = specificity protein 1.

Fig. 5 — Schematic representation showing how AOF might inhibit the IGF-II/II R-related signaling pathway that mediates Ang II-induced pathological hypertrophy, apoptosis, and remodeling in H9c2 cells. Ang II = angiotensin II; AOF = alpine Oxyphyllae fructus; IGF = insulin-like growth factor II.

In summary, this study found that Ang II significantly increased the IGF-II/II R-related signaling pathway that caused apoptosis, hypertrophy, and remodeling in H9c2 cells (Fig. 5). AOF reduced these events post-treatment, making it a candidate for treatment of cardiac hypertrophy, apoptosis, and ventricular remodeling in chronic cardiovascular disease.

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REFERENCES


