Pinocembrin Protects Cardiomyocytes Against Isoproterenol-Induced Hypertrophy

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Abstract

Cardiac hypertrophy is characterized by an increase in myocardial cell volume and extracellular matrix production. Persistent cardiac hypertrophy can cause dilated cardiomyopathy, heart failure, and even death. Pinocembrin (5,7-dihydroxyflavanone) is a type of flavonoid, extracted from propolis, that has antimicrobial, antioxidant, antiinflammatory, and anticancer properties. The results of the present study showed that pretreatment of isoproterenol (ISO)-treated H9c2 cardiomyocytes with pinocembrin reduced the messenger RNA levels of hypertrophic markers, including atrial natriuretic factor and βeta-myosin heavy chain, and inflammatory cytokines, such as tumor necrosis factor-α, interleukin-6, interleukin-1β, and interferon-γ, and also inhibited p65 phosphorylation and nuclear factor-kappa B (NF-κB) translocation. In addition, the activity of IκBα, an inhibitor of NF-κB, was increased while that of caspase-3 was reduced under these conditions. These results indicate that pinocembrin may inhibit ISO-induced myocardial hypertrophy by attenuating the NF-κB signaling pathway.

Keywords
flavonoid, pinocembrin, cardiac hypertrophy, nuclear factor-kappa B (NF-κB), isoproterenol

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Introduction

Cardiac hypertrophy is an important pathological basis for the development and progression of heart failure, representing a key step in the evolution of cardiac function from a compensatory to decompensated state and is an independent risk factor for cardiovascular complications.1,2 Cardiac hypertrophy is a multifaceted and complex clinical syndrome induced by a variety of stimuli that are associated with diverse pathological features, including reduced cardiac systolic and diastolic function, increased cardiomyocyte death and cardiac fibrotic remodelling.3,4 Therefore, it is important to suppress effectively cardiac hypertrophy to prevent the progression of cardiac failure. Isoproterenol (ISO), a β-adrenergic agonist, is used at specified doses to induce cardiac hypertrophy in animals to establish models of cardiac hypertrophy.5,7

Pinocembrin (Pi) is a type of flavonoid present in propolis that has antimicrobial, antiinflammatory, antiapoptotic, and antioxidant properties.8–10 One study showed that Pi can decrease the incidence of arrhythmia and the extent of myocardial infarction after acute cardiac ischemia/reperfusion injury by decreasing apoptosis and oxidative stress and increasing G×43 phosphorylation in the ischemic myocardium.12 Another study demonstrated that combination therapy with Pi and simvastatin was superior to simvastatin alone in inhibiting atherosclerosis in apolipoprotein E−/− mice.13 However, the effect of Pi on cardiac hypertrophy remains poorly understood. Thus, in the present study, we assessed the ability of Pi to protect cardiomyocytes against ISO-induced hypertrophy and evaluated whether nuclear factor-kappa B (NF-κB) signaling inhibition promotes Pi-mediated improvements in cardiac hypertrophy.

Materials and Methods

Materials

Pi was purchased from Shanghai Winherb Medical S & T Development, all tissue culture materials from GIBCO, all antibodies from Santa Cruz Biotechnology, and all other chemicals from Sigma.

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Cell Culture and Treatment

H9c2 cardiomyocytes (ATCC CRL-1446) were obtained from the Cell Bank of the Chinese Academy of Sciences and cultured as previously described.14 Cardiomyocytes were treated with either vehicle (0.1% dimethyl sulfoxide) or Pi (0.5–50 μM) in either the presence or absence of 20 μg/mL ISO. After pretreatment for 1 h with or without Pi, the cells were exposed to ISO for 24 h. In separate experiments, the cells were pretreated with the selective NF-κB antagonist pyrrolidine dithiocarbamate (PDTC) for 30 min prior to treatment with either vehicle (0.1% dimethyl sulfoxide) or Pi (25 μM) to examine the roles of estrogen receptor α and NF-κB in mediating the antiinflammatory and antiapoptotic effects of Pi.

Assessment of Cell Viability

Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, as previously described.14

Measurement of Cell Surface Area

To study the protective effect of Pi on ISO-induced hypertrophy, cells were plated in 6-well plates and treated as described above. The pretreated cells were washed twice with phosphate-buffered saline (PBS) prior to fixation in 4% paraformaldehyde, and after 10 min, the residual paraformaldehyde was removed with 3 5 min washes with 0.1% Triton X-100 (v/v, in PBS). Then, Actin-Tracker Green was added at a dilution of 1:100 in diluent (PBS supplemented with 2% bovine serum albumin and 0.1% Triton X-100). The cells were incubated in 6-well plates at room temperature for 30–60 min in the dark. Subsequently, excess Actin-Tracker Green solution was removed with 4 5 min washes with 0.1% Triton X-100 (see above) prior to visualization of the cells under a Nikon inverted microscope equipped with a polaroid digital camera at 200× magnification. Five random images were captured for each well, and 25 individual cell surface areas were measured using ImageJ (National Institutes of Health).

Caspase-3 Activity Assay

Caspase-3 activity was measured using a Fluorometric Assay Kit (BioVision) according to the manufacturer’s instructions. The fluorescence intensity of the samples was read using a Fluoroskan Ascent FL fluorimeter (Thermo Fisher Scientific) with excitation–emission wavelengths of 400 and 505 nm, respectively.

Real-Time Reverse Transcription–Polymerase Chain Reaction

Total RNA was extracted using TRIzol (Invitrogen), and ~2 μg of total RNA was reverse transcribed using a SuperScript First-Strand Synthesis System for reverse transcription–polymerase chain reaction (RT–PCR; Invitrogen). Cycle threshold values were obtained via real-time RT–PCR using a Power SYBR Green PCR Master Mix (Applied Biosystems), an iQ5 Real-Time PCR Detection System, and analysis software (Bio-Rad), as previously described.14 The primers were designed using Applied Biosystems Primer Express (version 2.0) (shown in Supplemental Table 1). The relative messenger RNA (mRNA) levels of target genes were normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and are presented as arbitrary units, with the value of the control group designated as 1.

Western Blot Analysis

Cell lysate preparation and Western blot analysis were performed as previously described.14 The membranes were probed with antibodies against target proteins, as well as an anti-GAPDH antibody as a loading control. The signals were quantified by scanning densitometry, and the results from each experimental group are expressed as the relative integrated density to that of the controls.

Statistical Analysis

The data are expressed as the means ± SE. The significance of the differences between means was assessed using Student’s t-test, and p values < .05 were considered to be significant. One-way analysis of variance with Bonferroni corrections was used to assess the significance for multiple comparisons. All statistical calculations were performed using SPSS (version 11.0).

Results

Effects of Pi on Cell Viability

To measure the cytotoxic effects of ISO and Pi (see chemical structure in Figure 1A), H9c2 cells were incubated with the indicated concentrations of ISO (20 μg/mL) for 24 h in the presence of 5, 10, 25, or 50 μM Pi. Then, cell viability was assessed using the MTT assay (expressed as a percentage of control). As shown in Figure 2A, cell viability gradually decreased as the ISO increased from 0 to 20 μM. Figure 2B shows that there was no significant difference in cell viability between the groups treated with various concentrations of Pi (0, 5, 10, 25, and 50 μM). Furthermore, treatment of H9c2 cells with ISO reduced cell viability to ~40% that of the control group (Figure 2C, P < .01), while Pi inhibited this decrease in a concentration-dependent manner (5, 10, and 25 μM) (Figure 1C, P < .05 or P < .01). Treatment with 25 μM Pi increased cell viability to ~90% of that of the control group. However, a higher concentration of Pi (50 μM) did not further improve cell viability.
Effects of Pi in Protecting Cardiomyocytes Against ISO-Induced Inflammation

No significant differences in the mRNA levels of tumor necrosis factor-α (TNF-α; Figure 3A), interleukin-6 (IL-6; Figure 3B), interleukin-1β (IL-1β; Figure 3C), and interferon-γ (IFN-γ; Figure 3D) were observed between the control and Pi-treated groups (Figure 3). However, the mRNA levels of TNF-α, IL-6, IL-1β, and IFN-γ notably increased when the cells were exposed to ISO, which was significantly suppressed in the Pi and ISO cotreatment group. In addition, the mRNA levels of these inflammatory factors were significantly decreased in response to pretreatment with losartan, which was used as a positive control (Figure 3). These data suggest that Pi pretreatment leads to the inhibition of ISO-induced myocardial inflammation.
levels compared to that observed in the control groups (data not shown). In contrast, p65 phosphorylation and IkBα degradation were significantly reduced in response to pretreatment with 25 μM Pi (Figure 4A and B). Accordingly, the phosphorylation of p65 and degradation of IkBα were also significantly reduced in response to pretreatment with losartan, which was used as a positive control (Figure 4C and D).

NF-κB Signaling is Associated With the Protective Effect of Pi on Hypertrophy in Cardiomyocytes

H9c2 cells were pretreated with PDTC, a specific inhibitor of NF-κB, for 30 min or with Pi or losartan for 1 h followed by treatment with ISO (20 μg/mL) for 24 h. As shown in Figure 4G, ISO clearly increased ANF mRNA levels compared to those observed in the control group, an effect that was inhibited in the groups cotreated with ISO and either Pi or losartan. These effects were similar to those observed in the group pretreated with PDTC, a specific inhibitor of NF-κB. As shown in Figure 4H, caspase-3 activity was significantly reduced upon Pi or losartan treatment and modestly reduced in response to treatment with PDTC (Figure 4H). Similar to the observed inhibition of caspase-3 activity, Pi and losartan also significantly inhibited the ISO-induced increase in the level of cleaved caspase-3, a key marker of apoptosis (Figure 4E and F), which was additional confirmation of suppressed caspase-3 activity upon Pi treatment. Treatment with Pi alone did not have a significant effect on either ANF expression or caspase-3 activity. These data suggest that the inhibitory effects of Pi on ISO-induced myocardial hypertrophy are closely associated with the inactivation of the NF-κB signaling pathway.

Discussion and Conclusions

Pi, the primary flavonoid in propolis, is believed to be responsible for its multiple pharmacological activities. The results of our recent studies have revealed that flavonoids extracted from propolis exert a protective effect against pathological cardiac hypertrophy through the phosphatidylinositol 3 kinase/protein kinase B (PI3K/AKT) signaling pathway. However, the effects of Pi on cardiomyocytes and its associated cellular/molecular mechanisms remain largely unknown. In the present study, we showed that Pi significantly attenuates ISO-induced myocardial hypertrophy in H9c2 cells. Furthermore, the cardioprotective effects of Pi were closely associated with the inhibition of NF-κB.

Cardiac hypertrophy, which occurs for a variety of reasons, such as hemodynamic overload, ischemic heart disease, and genetic cardiac defects, causes cardiac dysfunction through a complex sequence of pathophysiological changes, significantly increasing the morbidity and mortality rates of patients due to heart failure. The results of the present study showed that Pi enhances the viability and alleviates inflammation-mediated damage in cardiomyocytes by inhibiting relevant proinflammatory cytokines, including TNF-α, IL-6, IL-1β, and IFN-γ (Figure 3). Using the preestablished ISO-induced model of
cardiac hypertrophy in vitro, we observed that hypertrophic responses were enhanced in H9c2 cardiomyocytes in response to ISO treatment, as evidenced by the marked increase in cell surface area (Figure 1). Our data also showed that pretreatment with Pi could substantially reduce ISO-induced pathological cardiac hypertrophy (Figures 2 and 1). In this model, the levels of ANF and β-MHC mRNA were significantly higher with Pi pretreatment than with ISO treatment alone.

Interestingly, Pi promoted the activation of IκBα and reduced the phosphorylation of p65 (Figure 4). Based on the findings described above, we concluded that ISO is not only a potent stimulator of myocardial damage resulting from cardiac hypertrophy, but also a promoter of inflammation via IκBα degradation and p65 phosphorylation, leading to activation of the NF-κB signaling pathway. A previous study showed that Pi can inhibit the expression of lipopolysaccharide...
Figure 4. NF-κB signaling is associated with the effects of Pi on cardiomyocyte hypertrophy. (A, C) Cells were preincubated with (A) Pi (25 μM) or (C) losartan (10 μM) for 1 h followed by ISO treatment (20 μg/mL) for 24 h. Lysates were prepared from H9c2 cells, and immunoblots were probed for p-p65, total p65, and IκBα. (B, D) Quantitative analyses of NF-κB phosphorylation and IκBα degradation in H9c2 cells. (E) Cleaved caspase-3 protein levels in H9c2 cardiomyocytes were determined by western blot analysis. (F) Quantitative analyses of cleaved caspase-3. GAPDH was used as a loading control in all western blotting experiments. The data are presented as the means ± SE (n = 8 per group). *P < .05 versus ISO-treated cells. (G, H) Cells were preincubated with Pi (25 μM) or losartan (10 μM) for 1 h followed by ISO treatment (20 μg/mL) for 24 h. (A) Cells were preincubated with PDTC (a specific inhibitor of NF-κB) for 30 min prior to treatment with or without Pi or losartan (10 μM) for 1 h followed by ISO (20 μg/mL) for 24 h. ANF mRNA levels were determined by real-time RT–PCR. (B) Caspase-3 activity was measured using a fluorometric assay and expressed as the fold change compared to that observed in the control. The data are presented as the means ± SE (n = 6 per group). *P < .05 versus Cont; #P < .05 versus ISO-treated cells.

Abbreviations: NF-κB, nuclear factor-kappa B; Pi, pinocembrin; ISO, isoproterenol; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PDTC, pyrrolidine dithiocarbamate; ANF, atrial natriuretic factor; mRNA, messenger RNA; RT–PCR, reverse transcription–polymerase chain reaction; p-p65, phosho-p65; Cont, cells treated with vehicle (0.1% dimethyl sulfoxide); PC, positive control (10 μM losartan).
(LPS)-induced inflammatory mediators in cells, reduce the mortality from LPS-induced endotoxin shock in mice and alleviate endotoxin-induced acute lung injury by modulating NF-κB activation. Our data also showed that along with activating NF-κB, the levels of downstream proinflammatory mediators, such as TNF-α, IL-6, IL-1β, and IFN-γ, were increased. Among these mediators, the upregulation of TNF-α can directly lead to cellular apoptosis, as demonstrated by the activation of caspase-3 in cardiomyocytes (Figure 4E, F, and H). Furthermore, similar to Pi, the NF-κB inhibitor PDTC also partially inhibited the ISO-mediated expression of ANF mRNA and inhibited the ISO-mediated activation of caspase-3 in cardiomyocytes (Figure 4H). Thus, Pi likely protects cardiomyocytes against ISO-induced hypertrophy by blocking the NF-κB signaling pathway.

The underlying mechanism by which Pi improves cardiac hypertrophy needs to be further studied. Some investigators argue that in models of pathological hypertrophy, thyroid hormones can improve cardiac systolic function and transform pathological cardiac hypertrophy to physiological cardiac hypertrophy. Furthermore, a number of studies have also indicated that the insulin/insulin receptor/Akt signaling pathway can mediate cardiac hypertrophy. SIRT3 and SIRT6, which are known as sirtuins, have been recently reported to inhibit cardiac hypertrophy. In addition, the calcineurin signal transduction pathway has also been reported to play a crucial role in both physiological and pathological cardiac hypertrophy. As demonstrated in our present study and well documented by others, the production of inflammatory cytokines is closely linked to NF-κB signaling pathway activation in many pathological processes, including cardiac hypertrophy. In addition, a previous report showed that the relationship between NF-κB activity and cardiac hypertrophy is causal rather than coincidental.

In summary, the results of the present study demonstrate that pretreatment with Pi improves cell viability, reduces hypertrophic cell surface area, attenuates ISO-induced inflammatory cytokine production, and inhibits NF-κB activation in cardiomyocytes. Furthermore, Pi was shown to likely attenuate cardiac hypertrophy through the NF-κB signaling pathway. Although pretreatment with Pi significantly inhibited ISO-induced cardiac hypertrophy and inflammatory factor expression in cardiomyocytes, the results of the present study cannot be directly translated in vivo.

Based on the results of recent studies, to the best of our knowledge, Pi, a highly abundant flavonoid extracted from propolis, is a promising drug candidate for the clinical treatment of cardiac hypertrophy. As shown in our in vitro studies, both Pi and losartan have inhibitory effects on cardiomyocyte inflammation and apoptosis. Notably, both compounds directly suppressed p65 phosphorylation and caspase-3-mediated cellular apoptosis in cardiomyocytes. However, Pi cannot yet be used to treat cardiac hypertrophy in a clinical setting. Losartan, which was used as a positive control to inhibit cardiac hypertrophy, has been reported to suppress the cross-linking signals between myocytes and fibroblasts in the progression of cardiac hypertrophy. More than 30% of heart cells are noncardiomyocytes, such as fibroblasts, immune cells, and vascular endothelial cells. Thus, comprehensive in vivo animal studies are needed to validate further the efficacy of Pi in treating cardiac hypertrophy and translate these findings into clinical practice to improve treatment outcomes and reduce the suffering of hospitalized patients with cardiac hypertrophy.

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