Suppression of Isoproterenol-Induced Apoptosis in H9c2 Cardiomyoblast Cells by Daidzein through Activation of Akt

Wei-Syun Hu1, 2, Yueh-Min Lin3, 4, Wei-Wen Kuo5, Lung-Fa Pan6, 7, Yu-Lan Yeh3, 4, Yi-Hui Li3, Chia-Hua Kuo9, Ray-Jade Chen10, V. Vijaya Padma11, Tung-Sheng Chen8, 12, 9, and Chih-Yang Huang8, 13, 14, *

1School of Medicine, College of Medicine, China Medical University, Taichung 40402, Taiwan, R.O.C.
2Division of Cardiovascular Medicine, Department of Medicine, China Medical University Hospital, Taichung 40447, Taiwan, R.O.C.
3Department of Pathology, Changhua Christian Hospital, Changhua 50006, Taiwan, R.O.C.
4Jen-Teh Junior College of Medicine, Nursing and Management, Miaoli 35664, Taiwan, R.O.C.
5Department of Biological Science and Technology, China Medical University, Taichung 40402, Taiwan, R.O.C.
6Division of Cardiology, Armed Force Taichung General Hospital, Taichung 41152, Taiwan, R.O.C.
7Department of Medical Imaging and Radiological Sciences of Central Taiwan University of Science and Technology, Taichung 40601, Taiwan, R.O.C.
8Graduate Institute of Basic Medical Science, China Medical University, Taichung 40402, Taiwan, R.O.C.
9Department of Sports Sciences, University of Taipei, Taipei 10048, Taiwan, R.O.C.
10Department of Surgery, School of Medicine, College of Medicine, Taipei Medical University, Taipei 11031, Taiwan, R.O.C.
11Department of Biotechnology, Bharathiar University, Coimbatore-641 046, India.
12Biomaterials Translational Research Center, China Medical University Hospital, Taichung 40402, Taiwan, R.O.C.
13Graduate Institute of Chinese Medical Science, China Medical University, Taichung 40402, Taiwan, R.O.C.
14Department of Health and Nutrition Biotechnology, Asia University, Taichung 41354, Taiwan, Republic of China

Abstract

Increased serum norepinephrine level is one of pathological processes relating to heart disease (HD). Estrogens are considered as potential therapeutics for the treatment of HD; however, estrogen supplementation shows some side-effects, such as increasing the risk of developing breast, endometrial and ovarian cancers. This study investigated the cardio-protective effects of daidzein (Dai), a selective estrogen receptor modulator (SERM) from soy bean extract, in H9c2 cardiomyoblast cells treated with isoproterenol (ISO), a norepinephrine analog. In this in vitro model, H9c2 cells treated with Dai at different concentrations showed no statistical difference in cell viability. TdT-mediated digoxigenin-dUTP nick-end labeling (TUNEL) data and western blotting results indicated that Dai treated-H9c2 cells recovered from the damage induced by ISO. The recovery effects of Dai on ISO-induced damage were blocked by inhibition of Akt activation through adding Akt inhibitor. On the other hand, the fold
Heart disease (HD) is a multiple cardio-dysfunction process and a major cause of death worldwide. Several cardio-dysfunctions are associated with HD, including inflammation, hypertrophy, apoptosis and fibrosis (2, 11, 22). According to epidemiological reports, HD risk is lower in premenopausal women than in men of the same age (13). On the other hand, HD risk is increased in women with menopause. These evidences indicate that female hormones may partially play an important role in the cardio-dysfunction process (13).

Among the female hormones, estrogens show a cardiac protective role in vitro and in vivo studies (1, 5). Estrogens are the major sex steroids existing in three natural forms, including estrone (E1), 17β-estradiol (E2) and estriol (E3). Among these estrogens, E2 is most important due to its abundance and potency. Experimental results have indicated that estrogens regulate expression and activation of proteins related to cell survival, apoptosis suppression and other compensatory effects, leading to cardio-protection (23). Therefore, estrogen replacement therapy (ERT) in menopausal women has been considered as a therapeutic strategy for reducing HD risk (7, 18). Some reports stated that high-dose or long-term estrogen supplementation might activate cell proliferation and metastasis of breast, endometrial and ovarian cancers (8, 20). As a result, adequate supplementation of natural, botanical estrogen-like compounds that mimic E2 in reducing HD without adverse effects should be considered as an alternative to HRT in menopausal women.

Neurotransmitters, such as norepinephrine, can regulate heart function (4). Increased norepinephrine levels activate beta-adrenergic receptors and induce cellular signaling pathways activate apoptosis and suppress cell survival, leading to cardiomyocyte dysfunction (12, 15). Shizukuda et al. (21) and Saito et al. (19) reported that norepinephrine regulates calcineurine expression in heart cells, which then dephosphorylates the mitochondrial protein Bad, leading to mitochondrial membrane instability and the release of cytochrome C. This induces caspase 3 activation, resulting in cardiomyocyte programmed cell death. Furthermore, several papers reported cardiac protective effects of natural estrogen-like compounds on cardiomyocyte damage induced by isoproterenol (ISO) (3, 17), a norepinephrine analog which mimics hypertension in patients. Hu et al. (10) showed that genistein ameliorates cardiomyoblast hypertrophy induced by ISO through the p38-Erk1/2-JNK-NKκB signaling pathway. Maulik et al. (14) pointed out that ISO induces cardiac hypertrophy in rats through inducible nitric oxide synthase (iNOS) expression, and that cardiac hypertrophy can be suppressed by genistein treatment by inhibition of iNOS. These findings suggest that genistein shows cardiac protectives potentials in cardiac dysfunction induced by ISO. Taken together, norepinephrine exhibits pro-apoptotic effects on cardiomyocytes, and estrogens, including phytoestrogens, show cardio-protection both in vitro and in vivo. At present, the cardiac protective effects of daidzein (Dai), another natural estrogen-like compound from soy bean extract, has not been studied. Dai has as similar chemical structure as E2 and genistein (Fig. 1). The cardio-protective role of Dai in cardiac cells under ISO stress needs to be clarified.

We hypothesize that Dai protects cardiomyoblast cells from ISO-induced cell death (14) by activating pro-survival signaling pathways similar to E2 and genistein. To test our hypothesis, activation of pro-apoptotic and anti-apoptotic proteins was first investigated in H9c2 cardiomyoblasts treated with both Dai and ISO. We also determined which signaling pathway was responsible for the cardio-protective effects of Dai in the H9c2 cell model.

### Materials and Methods

#### Cell Culture

H9c2 cardiomyoblasts, a subclone of the original clonal cell line derived from embryonic BD1X rat heart tissues, obtained from the American Type Culture Collection (ATCC, CRL-1446) (Rockville, MD, USA)
were cultured in 10-cm culture dishes containing Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 100 μg/ml streptomycin, 100 μg/ml penicillin, 1 mM HEPS buffer, 2 mM glutamine and 10% Clontech fetal bovine serum in humidified air (5% CO2) at 37°C. Before treatment with indicated agents, H9c2 cardiomyoblasts, passage 32 to passage 40, were incubated overnight in serum-free essential medium.

**Cell Viability Assay**

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays were applied for measuring cell viability. Briefly, H9c2 cells (1 × 10^5 cells per well) were plated in 24-well plates. Different dosages of Dai were added to the wells and incubated for 24 h. The culture medium was then removed and 200 μl MTT solution (0.5 mg/ml) was added to each well. After 4 h incubation at 37°C, the MTT solution was removed and 150 μl dimethyl sulfoxide (DMSO) was added to each well. The absorbance was measured at 550 nm using an automated micro-plate reader.

**TUNEL (TdT-Mediated Digoxigenin-dUTP Nick-End Labeling) Assay**

The TUNEL method was carried out by using a commercial kit (Roche Molecular Biochemicals, Mannheim, Germany). To explore the Dai effects on the ISO-induced apoptosis, staining was performed according to the manufacturer’s protocol. The TUNEL assay was performed in a 96-well plate with 5,000 cells per well. TUNEL-positive cells were identified with a fluorescence microscope using an excitation wave-length in the 450–500 nm range and a detection wave-length in the 515–565 nm range (green). After TUNEL imaging, the specimen was stained with 4′,6-diamidino-2-phenylindole (DAPI) based on manufacturer’s protocol. Specimen was then read by using excitation wavelength of 358 nm and a detection wave-length of 461 nm (blue). The percentage of apoptotic cells was calculated by dividing the number of TUNEL-positive cells by the number of DAPI-positive cells visualized in the same field. Three independent experiments were then averaged and statistically analyzed.

**Western Blotting**

H9c2 cells with a cell density of 5 × 10^5 cells were plated onto 10-cm dish and incubated overnight at 37°C. Dosage (10^-8 M) of Dai and 1 μM kinase inhibitors including SP600125 (SP, JNK inhibitor), QZN (NFκB inhibitor), U0126 (Erk1/2 inhibitor), SB203580 (SB, p38 inhibitor) and LY294002 (LY, Akt inhibitor) were added to the dishes and pre-incubated for 1 h before ISO (50 μM) treatment. H9c2 cells were washed with cold PBS and resuspended in lysis buffer (50 mM Tris, pH 7.5, 0.5 M NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.5, 10% glycerol, 1 mM β-mercaptoethanol (BME), 1% IGEPAL-630) after treating ISO for 24 h with a proteinase inhibitor cocktail (Roche Molecular Biochemicals, New York, NY, USA) to extract total protein. After incubation for 30 min on ice, the supernatant was collected by centrifugation at 12,000 × g for 15 min at 4°C. The protein concentration was determined using the Bradford method. Samples containing 40 μg proteins were loaded to each well and analyzed using western blot analysis. Briefly, protein samples were separated by 12% SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore, Belford, MA, USA). Membranes were then blocked with a blocking buffer (20 mM Tris–HCl, 5% non-fat dry milk, pH 7.6, 0.1% Tween 20 and 150 mM NaCl) for at least 1 h at room temperature. Membranes were then incubated overnight with primary antibodies against Akt, phosphorylated Akt (p-Akt), Bad, phosphorylated Bad (p-Bad), Erk1/2, phosphorylated Erk1/2 (p-Erk1/2), caspase 3, caspase 9 and α-tubulin (all purchased from Santa Cruz Biotechnology, Inc., Paso Robles, CA, USA) in the above solution on an orbit shaker at 4°C. Following incubation with primary antibodies, membranes were then incubated with appropriate horseradish peroxidase-linked secondary antibodies (Santa Cruz Biotechnology, Inc.), and the membranes were observed under a cooled charge-coupled device (CCD) camera for detection of enzyme-based chemiluminescence in the membranes. Gels for western blots were analyzed by using ImageJ software (National institutes of health (NIH), Bethesda, MD, USA). In addition to Akt, Erk1/2 and Bad phosphorylation normalized with the respective total forms, bands in other western blots were normalized with an internal control, α-tubulin, and expressed as fold change.

**Statistical Analysis**

Data for TUNEL analysis and cell viability were...
expressed as mean ± SD (n = 3) and analyzed by using one-way ANOVA. Student’s t-test was applied for post-hoc test and statistical significance was considered at the level of $P < 0.05$. Correlation analysis was performed in Fig. 4 and two tailed F-test was applied for calculating significance. Statistical significance was considered at the level of $P < 0.05$.

**Results**

**H9c2 Cell Viability in the Presence of Dai**

Viability of H9c2 cardiomyoblasts in the presence of Dai is shown in Fig. 2. Compared to the control (H9c2 cells in the absence of Dai), no significant changes were observed in the presence of different Dai con-
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The cell viability results exhibited that treatment Dai was not cytotoxic to the H9c2 cells at the concentrations tested. The Dai treatment dosage for H9c2 cells used in subsequent experiments in this study was $10^{-8}$ M.

**TUNEL Analysis of H9c2 Cells in the Presence of Dai**

The TUNEL assay results to investigate H9c2 cell apoptosis in the presence of Dai or ISO are shown in Fig. 3. Compared to the control (upper panel, first column), the TUNEL-positive signal (apoptotic bodies, shown as green fluorescence) for ISO-treated H9c2 cells (upper panel, third column) increased significantly.

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**Fig. 4.** Western blot analysis for H9c2 cells including control (con), ISO (50 μM) and/or Dai. (A) activation of Akt, Bad and Erk1/2, (B) correlation for p-Akt/Akt, p-Bad/Bad and p-Erk/Erk vs. treatment time, and (C) expression and activation of caspase 3 and caspase 9.

**centrations (ranging from $10^{-10}$ to $10^{-5}$ M, $P > 0.05$).**

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In contrast, the TUNEL-positive signals were significantly decreased when different concentrations of Dai were added to the H9c2 cells in the presence of ISO challenge (Fig. 3A). The quantified results of Fig. 3A are presented in Fig. 3B, which showed statistically significant differences ($P < 0.01$) for ISO, ISO + Dai (10$^{-10}$ M) and ISO + Dai (10$^{-9}$ M) when compared to the control. Further significance could be observed in groups ISO + Dai (10$^{-8}$ M) ($P < 0.05$), ISO + Dai (10$^{-7}$ M) ($P < 0.05$), ISO + Dai (10$^{-6}$ M) ($P < 0.05$) and ISO + Dai (10$^{-5}$ M) ($P < 0.01$) when compared to the ISO treatment group.

**Protein Activation in H9c2 Cells in the Presence of Dai and ISO**

Western blotting approach was performed for investigating the levels of Akt, Erk1/2 and Bad activation in H9c2 cells in the presence of both ISO and Dai treatments, as represented by phosphorylation of these proteins. Expression levels of p-Akt, Akt, p-Bad, p-Erk1/2 and Erk1/2 were found to be increased in H9c2 cells treated with Dai for 1 h when compared to the control (con) (Fig. 4A). After 1 h, most of the protein bands decreased in intensities except for Erk1/2. The fold changes of p-Akt/Akt, p-Bad/Bad, p-Erk1/Erk1 and p-Erk2/Erk2 normalized with controls are shown in the Fig. 4A. In addition, correlation analysis between p-Akt/Akt, p-Bad/Bad, p-Erk1/Erk1 and p-Erk2/Erk2 vs. treatment time showed that p-Erk1/Erk1 and p-Erk2/Erk2 correlations were significantly ($P < 0.05$) (Fig. 4B). It was further observed that some protein levels were increased in ISO-treated H9c2 cells, including the active form of caspase 9 and caspase 3 when compared to the control (Fig. 4C, columns 1 and 2). By contrast, the caspase 9 and caspase 3 protein levels were suppressed after addition of Dai (10$^{-8}$ M) into ISO-treated H9c2 cells (Fig. 4C, column 3). The fold change for caspase 9 and 3 for control, ISO and ISO + Dai were 1, 1, 0.7, and 1, 1.5 and 0.7, respectively.

**Caspase Activation in H9c2 Cells in the Presence of Dai, ISO and Kinase Inhibitors**

In order to investigate which kinase pathways are involved in the Dai protective effects in ISO-treated H9c2 cells, inhibitors of signal pathways were tested (Fig. 5). The fold changes were expressed in the Figs. 5, A and B.

**Discussion**

We investigated the activation of pro- and anti-apoptotic proteins by Dai. Our data showed that compared to the ISO-treatment, the number of apoptotic cells was significantly decreased after treating the H9c2 cells with various Dai concentrations. These TUNEL images illustrate that Dai treatment decreased ISO-induced apoptosis in H9c2 cells in a dose-dependent manner, leading to cardiomyoblasts protection under stress.

Our results further indicated that the activation of Akt was increased in a time-dependent manner in H9c2 cells after treatment with Dai, while Bad was inactivated (Figs. 4, A and B). Furthermore, activation of Erk1 and Erk2 (increased p-Erk1/Erk1 ratio) was observed in 1 h and then decreased; p-Erk2/Erk2 was activated in 30 min before decrease. These findings illustrate that Dai is capable of activating survival proteins, but inhibiting pro-apoptotic proteins in H9c2 cells. ISO induced activation of pro-apoptotic caspases 9 and 3, which was reversed by Dai treatment (Fig. 4B). Furthermore, we found that such protective effects of Dai no longer existed by adding the Akt inhibitor. This finding suggests that Akt activation plays a central role in mediating cardio-protection exerted by Dai against ISO-induced myocardial injury (Fig. 5).

Fan et al. (6) pointed out that lipopolysaccharide (LPS) treatment induces inflammation in H9c2 cells through expression of toll-like receptor 4 (TLR4). Besides, upregulation of TLR4 accompanies suppression of survival markers, such as insulin-like growth factor 1 (IGF-1), phosphoinositide 3-kinase (PI3K) and Akt, leading to cardiac cell apoptosis. Estrodiol/estrogen receptor α (E2/ERα) treatment is capable of suppressing LPS-induced TLR4 expression, resulting in reduction...
leading to increase of p-Bad and decrease of intrinsic apoptosis in ISO-damaged H9c2 cells.

The role of Akt expression is confirmed by using kinase inhibitors in this study. Although Akt expression plays a central role in Dai cardio-protection for H9c2 cardiomyoblasts in the presence of ISO stress, siRNA should be used to confirm the role of Akt in this in vitro model because it is unclear if LY2 is specific to inhibition of Akt expression. Furthermore, the relationship between Dai and ER should be investigated, and an animal model should be designed in a future study.

The protective role of Dai in ISO-treated H9c2 cells can be summarized as follows: [1] ISO triggers programmed cell death by mediating activation of caspase 9 and 3. [2] Dai upregulates the activation of Akt, then inactivates Bad, leading to suppressed apoptosis signaling induced by the Bad-caspase 9-caspase 3 axis. The key findings in this study are summarized in Fig. 6. Other signaling pathways, such as cardiac hypertrophy, fibrosis or inflammation, can also be investigated in the future to further understand the mechanism of Dai in the cardioprotective effects.

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