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Research Paper

The involvement of endoplasmic reticulum stress in flavonoid-induced protection on cardiac cell death caused by ischaemia/reperfusion

Do-Sung Kim[a], Dae-Young Kwon[b], Myung-Sunny Kim[a], Hye Kyung Kim[a], Yong Chul Lee[c,d], Seong Ju Park[c,d], Wan Hee Yoo[c,d], Soo-Wan Chae[a], Myoung-Ja Chung[e], Hyung-Ryong Kim[f] and Han-Jung Chae[a,d]

[a]Department of Pharmacology and Institute of Cardiovascular Research, and [c]Department of Internal Medicine, Medical School, Chonbuk National University, Jeonju, Chonbuk, [b]Korea Food Research Institute, Kyongi-do, [d]Research Center for Pulmonary Disorders, Chonbuk National University Hospital, Jeonju, Chonbuk, [e]Department of Pathology, Chonbuk National University, Jeonju, Chonbuk and [f]Department of Dental Pharmacology, School of Dentistry, Wonkwang University, Iksan, Chonbuk, Republic of Korea

Abstract

Objectives We have investigated whether endoplasmic reticulum stress and Bcl-2 proteins were linked to the protective effect exerted by flavonoids on ischaemia/reperfusion-induced cardiac damage.

Methods Cell viability and immunoblotting were performed.

Key findings H9c2 cardiac muscle cells were exposed to flavonoids such as biochanin A, daidzein, genistein, luteolin, quercetin and rutin, followed by ischaemia 12 h/reperfusion 4 h. The flavonoids protected against cell death induced by ischaemia/reperfusion. Flavonoid treatment significantly increased the expression level of the anti-apoptotic protein, Bcl-2, but decreased that of the proapoptotic protein, Bax. The flavonoids down-regulated the expression levels of endoplasmic reticulum stress proteins, glucose-regulated protein-78, activating transcription factor 6α, X-box binding protein 1, inositol-requiring protein-1, phosphor-eukaryotic initiation factor 2α, and C/EBP-homologous protein.

Conclusions This study suggested that the protective mechanisms of flavonoids included regulation of Bcl-2/Bax proteins as well as the endoplasmic reticulum stress proteins.

Keywords apoptosis; endoplasmic reticulum stress; flavonoids; ischaemia/reperfusion

Introduction

Cardiovascular disease is one of the leading causes of death in Korea and North America. One of the most important presentations of cardiovascular disease is ischaemia, which leads to tissue hypoxia, cellular necrosis, apoptosis and organ dysfunction in severe situations. In ischaemic conditions, reduced blood flow resulting from an arterial occlusion or cardiac arrest causes tissue hypoxia and hypoglycaemia, which initiate protein misfolding and endoplasmic reticulum (ER) stress.[1,2] Reperfusion of the affected tissues then triggers oxidative stress, with the production of nitric oxide (NO) and other reactive oxygen species (ROS) that result in protein misfolding-ER stress. ER stress triggers complex adaptive or proapoptotic signalling defined as the unfolded protein response (UPR). This is involved in several pathophysiological processes.[2–5] Since protein folding is highly redox-dependent, the convergence between ER stress and oxidative stress is interesting.[6] ROS production and oxidative stress are not only coincidental to ER stress, but are integral UPR components, being triggered by distinct types of ER stressors and contributing to the proapoptotic, as well as proadaptive UPR signalling.[2–7] In ER stress processes, glucose-regulated protein-78 (Grp78) is released from the transmembrane signalling proteins PERK (protein kinase-like endoplasmic reticulum kinase), inositol-requiring protein-1 (Ire1) and activating transcription factor 6 (ATF-6). Upon removal of Grp78, PERK oligomerizes in ER membranes, phosphorylating and inactivating phosphor-eukaryotic initiation factor 2α (eIF2α). X-box binding protein 1 (XBP-1) and ATF-6 are transcription factors that increase C/EBP-homologous protein (CHOP) expression during ER stress.[8] Activated Ire1 initiates unconventional splicing of the mRNA encoding an isoform of

Correspondence:
ChaeHan-JungDepartment of Pharmacology and Institute of Cardiovascular Research, Medical School, Chonbuk National University, Jeonju, Chonbuk, Republic of Korea.
E-mail: hjchae@chonbuk.ac.kr

Hyung-Ryong Kim and Han-Jung Chae contributed equally to this work.

XBP-1 protein that induces expression of CHOP. ATF-6 is cleaved during ER stress and its cytosolic domain translocates to the nucleus. Severe ER stress causes cell death to occur, which involves expression of pro/anti-apoptotic Bcl-2 family proteins and caspase activation. ER stress signalling has been studied already in the field of cardiac ischaemia.

Flavonoids are naturally occurring polyphenolic compounds found in plants, including fruits, vegetables, wines, teas and cocoa and have a variety of biological activities. Flavonoids have in common phenolic rings with variations in the number and arrangement of hydroxyl groups as well as the nature and extent of alkylation or glycosylation of these groups.[9–12] Some of these inhibit ROS generation during heart ischaemia/reperfusion.[13] In one study, perfusing hearts with quercetin for 30 min before ischaemia reduced malondialdehyde levels in heart tissues after reperfusion. Furthermore, a flavonoid, kaempferol, regulated ischaemia/reperfusion-induced alteration of cardiac haemodynamic factors and damage, probably through the regulatory effect of ER stress responses.[14] Although flavonoids can protect the heart from ischaemia/reperfusion-induced injury by scavenging ROS, it is still controversial as to whether the antioxidant activity of flavonoids are solely responsible for their protective effect or whether flavonoid-induced consequential effects such as antioxidant protein expression or another kinase activation are involved.

In this study, we have investigated the effect of several flavonoids for their protective functions on ischaemia/reperfusion-associated cardiac cell death. We attempted to demonstrate a possible link between ER stress and ischaemia/reperfusion-induced cardiac cell death, showing the expression of Bcl-2 family proteins and the activity of caspase.

Materials and Methods

Materials

Biochanin A, daidzein, genistein, luteolin, quercetin and rutin were purchased from Sigma Chemical Co (St Louis, MO, USA). Anti-Bcl-2, Bax, Grp78, CHOP, Ire-1α, cleaved ATF-6 and spliced XBP-1 antibodies were acquired from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-actin antibody was supplied by Sigma Chemical Co. The plastic culture-ware was purchased from Falcon Inc. (Miami, FL, USA). Dulbecco's modified essential medium (DMEM), fetal bovine serum (FBS) and the other tissue culture reagents were obtained from Gibco (Grand Island, NY, USA).

Simulated ischaemia/reperfusion of cardiomyocytes

Ischaemia/reperfusion was performed using a modified protocol described elsewhere.[15] To mimic the ischaemia/reperfusion process *in vitro*, H9c2 cells were incubated in stimulated ischaemia buffer: DMEM base (Sigma) at pH 6.2, deprived of glucose and serum for 12 h, with a constant stream of water-saturated 5% CO_2–95% N_2 (< 1% O_2). After 12-h exposure to the stimulated ischaemia buffer, the cells were subjected to reperfusion in a water-saturated atmosphere of 5% CO_2–95% air for 4 h. The ischaemia buffer was replaced with 0.3 ml reperfusion medium. H9c2 cells were then assessed using a microscope for dead cells using Trypan blue exclusion.

Cell viability assay

H9c2 cells were then assessed using a microscope for dead cells by Trypan blue exclusion. Cell viability was calculated by dividing the nonstained (viable) cell count by the total cell count. The number of cells was determined by averaging the number of cells in four squares and by multiplying this average by a dilution factor.

Determination of caspase-3 activity

H9c2 cells (3×10^6) were washed with phosphate-buffered saline (PBS) and incubated for 30 min on ice with 100 ml lysis buffer (in mM: 10 Tris-HCl, 10 NaH_2PO_4/$NaHPO_4$, pH 7.5, 130 NaCl, 1% Triton1 X-100, and 10 sodium pyrophosphate). Cell lysates were spun down, supernatants were collected, and protein concentrations were determined using the bicinchoninic acid (BCA) method. For each reaction, 30 μg protein was added to 1 ml freshly prepared protease assay buffer: 20 mM N′-2-hydroxyethylpiperazine-N′-2 ethanesulphonic acid (HEPES) pH 7.5, 10% glycerol, 2 mM dithiothreitol) containing 20 mM acetyl Asp-Glu-Val-Asp 7-amido-4-methylcoumarin (AC-DEVD-AMC; Sigma-Aldrich, St Louis, MO, USA). Reaction mixtures without cellular extracts were used as negative controls. Reaction mixtures were incubated for 1 h at 37°C and the aminomethyl-coumarin liberated from AC-DEVD-AMC was determined by spectrofluorimetry (Hitachi F-2500) at 380 $nm^{excitation}$ and 400–550 $nm^{emission}$. Readings were corrected for background fluorescence.

Western blotting

Western blotting analysis was performed using a protocol described elsewhere.[14] Total protein was electrophoretically resolved using precast 4–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gradient gels and transferred onto nitrocellulose membranes. After blocking with 1.5% bovine serum albumin (BSA), the membranes were incubated with the indicated primary antibody, followed by a secondary antibody. Samples were finally detected using enhanced chemiluminescence (Amersham Pharmacia Biotech, Amersham, UK).

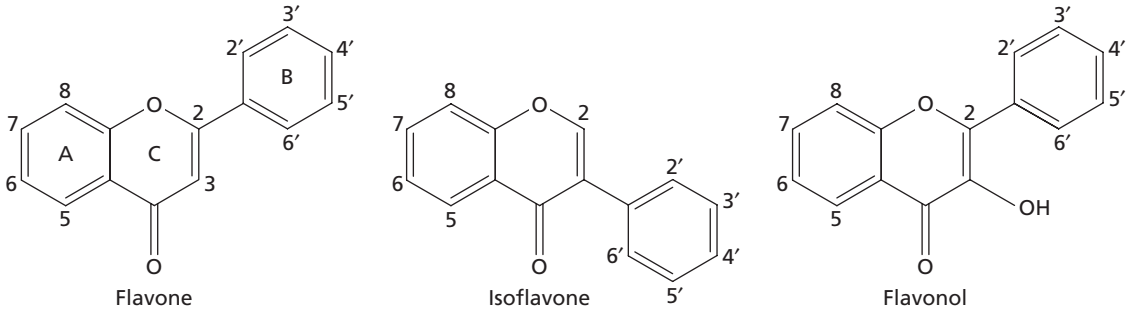
Statistical analysis

The data was analysed using an analysis of variance in the dose–response experiments, and two-tailed Student's *t*-tests. A P value < 0.05 was considered significant. In each case, the statistical test used is indicated, and the number of experiments is stated individually in each figure legend.

Results

Effect of flavonoids on ischaemia/reperfusion-induced apoptosis in cardiomyocytes

Flavonoids include various chemical structures such as flavone, flavonol and isoflavone (Figure 1).[16] Among the flavonoids, biochanin A, daidzein, genistein, luteolin, quercetin and rutin were selected for the study of the cardiac protective effect against ischaemia/reperfusion, based on flavonoid studies in



Class	Derivatives	Substituents					
		3	5	7	3′	4′	5′
Flavones	Luteolin	H	OH	OH	OH	OH	H
Isoflavones	Genistein	H	OH	OH	H	OH	H
	Daidzein	H	H	OH	H	OH	H
	Biochanin A	H	OH	OH	H	OCH_3	H
Flavonols	Quercetin	OH	OH	OH	OH	OH	H
	Rutin	ORu	OH	OH	OH	OH	H

Figure 1 Chemical structures of the flavonoids used in the study

ischaemia.[13,14,17–24] Toxicity was tested using a 24-h incubation in H9c2 cells. Protection was tested by pretreatment before ischaemia/reperfusion (Tables 1, 2). The best concentrations of flavonoids were selected based on lack of toxicity and protective effect: 10 μM biochanin A; 1 μM daidzein; 25 μM genistein; 10 μM luteolin; 50 μM quercetin; 50 μM rutin. The cells were exposed to the selected concentrations of the flavonoids followed by ischaemia 16 h/reperfusion 8 h, an in-vitro ischaemia/reperfusion model, which induced stable and reproducible injury.[14] Figure 2a shows the effect of flavonoids on the cytotoxicity exerted by ischaemia/reperfusion in the cardiac muscle cells. The protective effects against ischaemia/reperfusion were similar among the flavonoids. Figure 2b shows the protective effects of the flavonoids using light microscopy.

Effect of flavonoids on the ischaemia/reperfusion-associated expression of Bcl-2 and Bax

To understand the mechanisms of the flavonoids, we first examined whether the expression of Bcl-2 proteins (anti-apoptotic Bcl-2 and proapoptotic Bax) on ischaemia/reperfusion was modulated by the flavonoids. H9c2 cells were pretreated with 10 μM biochanin A, 1 μM daidzein, 25 μM genistein, 10 μM luteolin, 50 μM quercetin or 50 μM rutin for 30 min and then exposed to ischaemia/reperfusion for 12 h/4 h, which maintained cell viability. Ischaemia/reperfusion increased Bcl-2 levels, but decreased Bax levels (Figure 3). All the flavonoids markedly increased the expression of Bcl-2, but reduced the expression of Bax. β-Actin was used as a loading control to confirm the nature of the changes in Bcl-2 and Bax levels.

Effect of flavonoids on ischaemia/reperfusion-induced caspase-3 activation

Caspase cascade activation is critical for apoptotic initiation in many biological systems.[25] To determine whether caspase-3 contributed towards ischaemia/reperfusion injury, we monitored its catalytic activity. The catalytic activity of caspase-3 significantly increased up to 5-fold after ischaemia/reperfusion. Flavonoids significantly decreased the activation of the executor caspase, caspase-3 (Figure 4).

Effect of flavonoids on ischaemia/reperfusion-induced endoplasmic reticulum stress signalling proteins

We measured the expression levels of ER stress-associated proteins. Protein expression transiently increased for Ire-1α, XBP-1 and ATF-6 (Figure 5). P-eIF2-α, Ire-1α, and ATF-6 sharply increased early (0.5 or 1 h) but decreased after 4 or 8 h. We subsequently used 1-h time points for expression of ER stress proteins. Flavonoids changed the levels of Grp78 and phosphorylation of eIF-2α, and decreased the level and splicing of XBP-1 protein and cleaved ATF-6 (Figure 6). This suggested that the flavonoids regulated ATF-6 processing, inhibited selected Ire-1α activity, and regulated the expression of proapoptotic CHOP.

Table 1 Effects of various flavonoids on H9c2 cell viability

Flavonoid	Concentration (μM)	Cell viability (%)
Control (no flavonoid)	–	98.3 ± 2.5
Biochanin A	1	97.3 ± 1.5
	5	98.3 ± 2.1
	10	96.8 ± 1.8
	25	95.9 ± 3.7
	50	75.3 ± 5.5
Daidzein	0.1	96.9 ± 3.1
	0.5	97.3 ± 2.7
	1	96.8 ± 1.9
	5	93.9 ± 5.1
	10	75.3 ± 5.5
Genistein	1	98.3 ± 3.5
	5	96.3 ± 2.7
	10	95.8 ± 1.2
	25	95.9 ± 2.9
	50	87.3 ± 4.3
Luteolin	1	96.3 ± 3.3
	5	97.3 ± 2.4
	10	98.8 ± 3.2
	20	97.9 ± 2.9
	50	96.3 ± 3.1
Quercetin	1	97.3 ± 1.6
	5	97.3 ± 3.1
	10	98.1 ± 1.2
	50	95.8 ± 2.3
	100	85.9 ± 7.2
Rutin	1	95.3 ± 2.5
	5	96.3 ± 3.3
	10	95.8 ± 4.3
	50	97.3 ± 3.1
	100	98.3 ± 2.5

Table 2 Effects of various flavonoids on ischaemia/reperfusion-induced H9c2 cell viability

Ischaemia/reperfusion + flavonoid	Flavonoid concentration (μM)	Cell viability (%)
Control	–	98.3 ± 2.3
Ischaemia/reperfusion (no flavonoid)	–	40.4 ± 6.3$^{\#}$
Ischaemia/reperfusion + biochanin A	1	45.3 ± 1.5
	5	63.8 ± 2.1*
	10	80.3 ± 2.1*
	25	78.9 ± 6.3*
	50	72.3 ± 4.7*
Ischaemia/reperfusion + daidzein	0.1	52.9 ± 8.1
	10.5	62.8 ± 4.2*
	1	71.6 ± 2.1*
	5	68.5 ± 5.1*
	10	50.3 ± 5.5
Ischaemia/reperfusion + genistein	1	48.9 ± 6.1
	5	63.0 ± 5.7*
	10	73.8 ± 3.2*
	25	77.5 ± 1.2*
	50	75.3 ± 3.9*
Ischaemia/reperfusion + luteolin	1	49.0 ± 2.9
	5	62.8 ± 5.3*
	10	69.1 ± 2.5*
	20	70.1 ± 6.3*
	50	65.3 ± 5.9*
Ischaemia/reperfusion + quercetin	1	52.4 ± 3.9*
	5	58.7 ± 7.2*
	10	70.8 ± 3.2*
	50	75.4 ± 1.8*
	100	66.7 ± 5.4*
Ischaemia/reperfusion + rutin	1	58.2 ± 5.9*
	5	63.8 ± 4.1*
	10	70.3 ± 6.4*
	50	81.6 ± 0.5*
	100	79.8 ± 3.2*

$^{\#}P < 0.05$ compared with control; $^{*}P < 0.05$ compared with ischaemia/reperfusion.

Discussion

This study has shown that flavonoids significantly regulated ischaemia/reperfusion-induced cell death. The mechanism of protection included regulation of the Bcl-2 and Bax proteins, inhibition of caspase-3 activation, and inhibition of ER stress protein activation.

In ischaemia/reperfusion-associated biological changes, ROS production is a detrimental source of cell death. ROS might modulate the cellular distribution and content of Bax and Bcl-2.[14] Our results showed that ischaemia/reperfusion promoted the levels of Bax but decreased the levels of Bcl-2 (Figure 3), which probably led to dysfunction of the mitochondrial membrane. Bax expression increases are followed by the translocation of this protein into the mitochondria.[15] The mitochondrial translocation of Bax can cause the release of cytochrome *c* and induce cell death. The anti-apoptotic protein Bcl-2 prevented apoptosis by scavenging oxygen-derived free radicals inside cells.[14,26]

Flavonoids changed the expression of Bcl-2 and Bax (Figure 3). These results were similar to another report showing that flavonoids altered the expression or the ratio of the apoptosis-associated central checkpoint proteins, Bcl-2 and Bax.[17,27] In addition, flavonoids decreased the ischaemia/reperfusion-induced activation of the apoptosis executor caspase-3 in cardiac muscle cells (Figure 4), clearly suggesting a regulatory role of flavonoids on apoptosis.

In organs such as the brain and heart, ischaemic conditions involving mitochondrial Bcl-2 triggered ER stress leading to cell death.[28,29] This study supported this, demonstrating the up-regulation of ER stress proteins after ischaemia/reperfusion (Figure 5). A cardiology study using human heart samples suggested that chronic congestive heart failure may be linked to ER stress proteins.[30] ER stress signalling is triggered by a range of pathogenic conditions, including ischaemia/reperfusion.[30,31] The expression levels of the UPR-related proteins, Grp78, XBP-1, cleaved ATF-6, Ire-1α and CHOP, were markedly regulated by the addition of flavonoids (Figure 6).

Conflicting ideas of the adaptive and apoptotic role of ER stress proteins upon exposure to stress still exist.[29] The UPR is an adaptive response, aiming to restore ER homeostasis and protect the cells from stress. The importance of the UPR in protecting the stressed heart was first shown by Martindale *et al*.[30], who demonstrated that the tamoxifen-regulated form of ATF-6 protected against ischaemia/reperfusion injury. The cytoprotective responses of chaperone proteins induce or

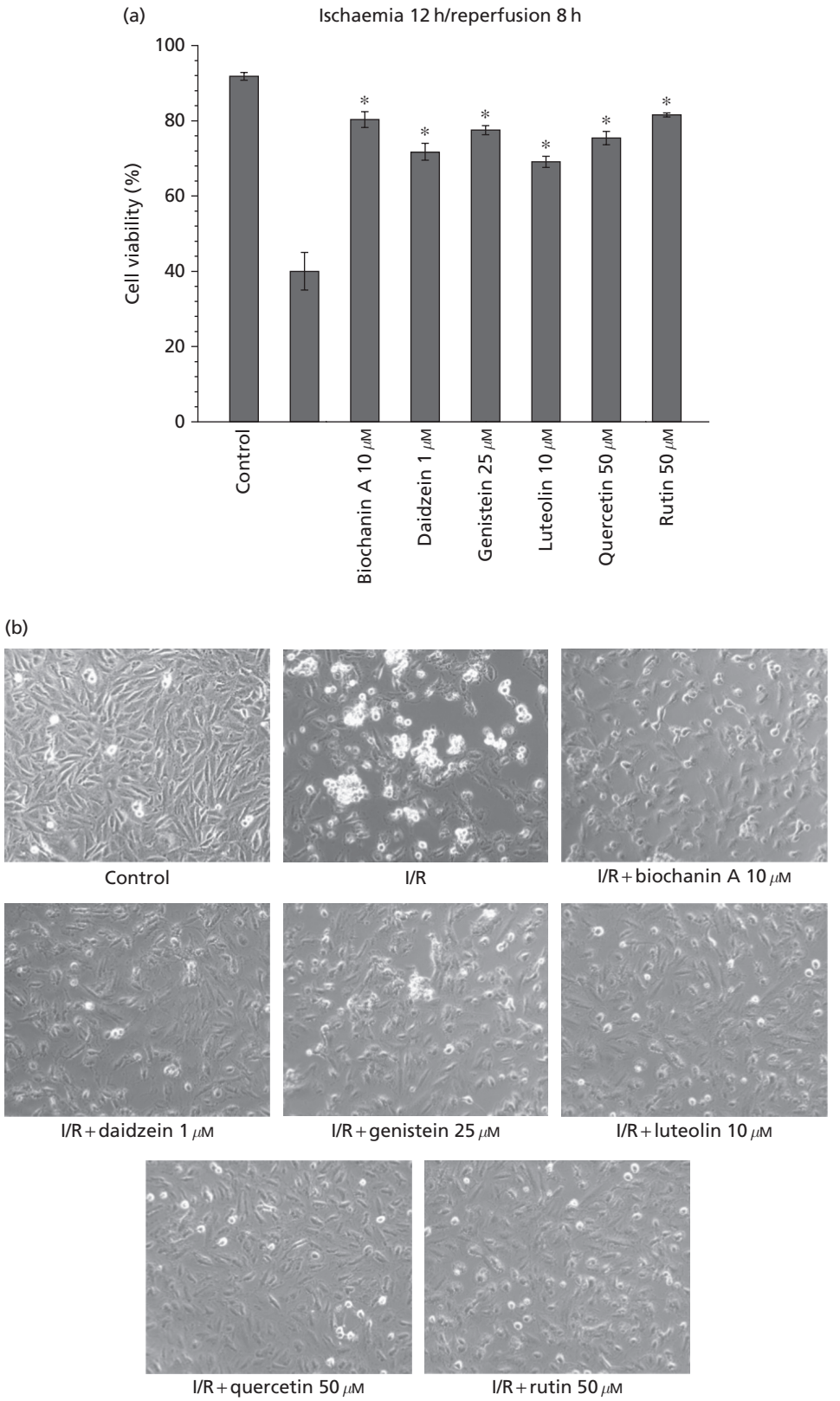


Figure 2 Flavonoids offered protection from ischaemia/reperfusion-induced cardiac cell death. The H9c2 cells were exposed to ischaemia 12 h/reperfusion 8 h in the presence or absence of the indicated concentrations of flavonoids. Cell viability was measured using Trypan blue methods. (a) The data shown represents means ± SE ($n = 5$). *$P < 0.05$ significantly different from the ischaemia/reperfusion-exposed H9c2 cells. (b) Damaged cell morphology was observed using optical microscopy (× 200). I/R, ischaemia/reperfusion.

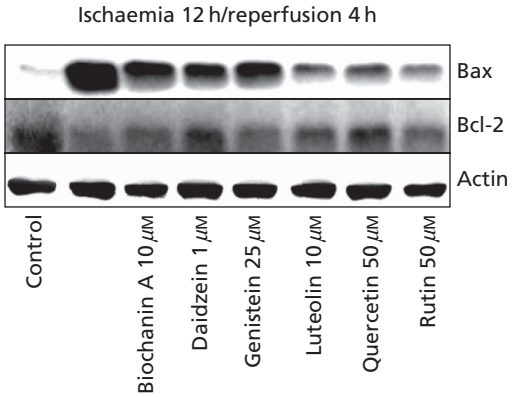


Figure 3 Flavonoids regulated the expression of Bcl-2 and Bax. H9c2 cells were pretreated with the indicated concentrations of flavonoids for 30 min and exposed to ischaemia 12 h/reperfusion 4 h. Expressional changes in the Bcl-2 proteins were studied by Western blotting analysis using anti-Bcl-2, anti-Bax, and anti-β-actin (loading control) antibodies. Immunoreactive bands were visualized by LAS-3000 (Fuji-film).

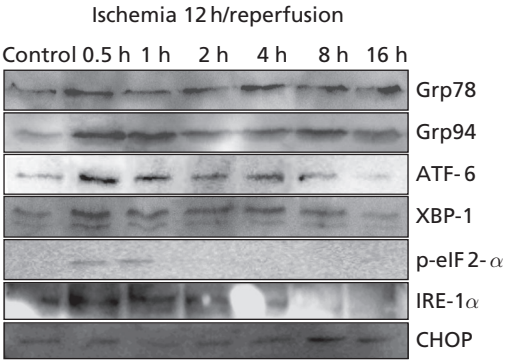


Figure 5 Ischaemia/reperfusion induced the expression of endoplasmic reticulum stress proteins. H9c2 cells were exposed to ischaemia 12 h and then reperfusion during the indicated times. The endoplasmic reticulum stress proteins, which included glucose-regulated protein-78 (Grp78), activating transcription factor 6α (ATF-6), X-box binding protein 1 (XBP-1), phosphor-eukaryotic initiation factor 2α (p-eIF2α) inositol-requiring protein-1α (Ire-1α), and C/EBP-homologous protein (CHOP), were determined by Western blotting.

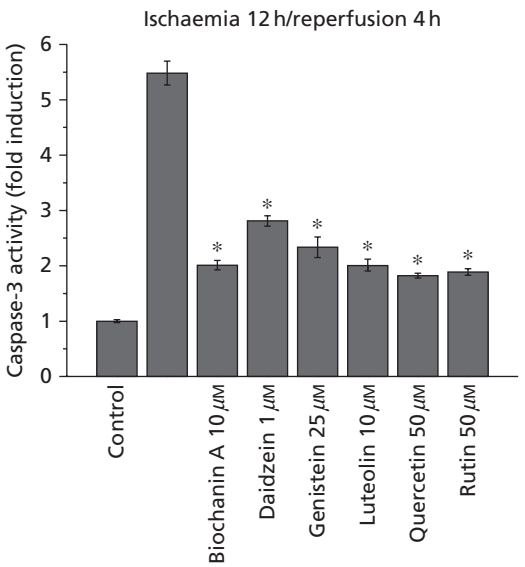


Figure 4 Flavonoids inhibited the activation of caspase-3 induced by ischaemia/reperfusion. Cells were pretreated with the indicated concentrations of flavonoids for 30 min and exposed to ischaemia 12 h/reperfusion 4 h. Caspase-3 activity in the cytosolic fraction was monitored by measuring the absorbance of the cleaved fluorogenic substrate, acetyl Asp-Glu-Val-Asp 7-amido-4-methylcoumarin. Data represent means ± SE (n = 3). $^{*}P < 0.01$; significantly different from ischaemia/reperfusion exposed H9c2 cells.

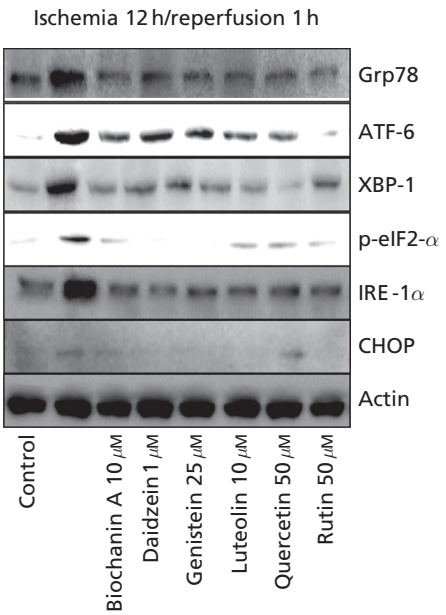


Figure 6 Flavonoids regulated the expression of endoplasmic reticulum stress proteins in ischaemia/reperfusion-exposed cardiac cells. H9c2 cells were pretreated with the indicated concentrations of flavonoids for 30 min and then exposed to ischaemia 12 h/reperfusion 1 h. Expression levels of endoplasmic reticulum stress proteins, glucose-regulated protein-78 (Grp78) and 94, activating transcription factor 6α (ATF-6), X-box binding protein 1 (XBP-1), phosphor-eukaryotic initiation factor 2α (p-eIF-2α), inositol-requiring protein-1α (Ire-1α) and C/EBP-homologous protein (CHOP) and actin were analysed.

suppress translation such that the amount of misfolded protein is reduced and cell survival is enhanced.[30,31] However, the treatment of flavonoids decreased the UPR (Figure 5), suggesting some other possibilities. For example, flavonoids themselves could increase the expression or activity of ER stress regulating proteins such as Bax inhibitor-1 (BI-1).[2] We were unable to analyse the expression levels of BI-1 protein due to the absence of high quality antibodies. Thus, the development of antibodies against this protein for immunoblotting analysis would help resolve the role of ER stress proteins in the regulatory mechanism of flavonoids.

High loads of misfolded protein induced caspase-mediated apoptosis.[32,33] Upon severe ER stress, the initiation of the

apoptotic processes is promoted by transcriptional induction of CHOP or by the activation of c-Jun NH_2-terminal kinase/stress-activated protein kinase- or caspase-12-dependent pathways.[32,33] CHOP is induced at the transcriptional level mainly when the ER is stressed.[34,35] The overexpression of CHOP leads to growth arrest and apoptosis.[36] This is a strong indication of ER-initiated apoptosis being promoted. Caspase-12 is located on the ER membrane and is activated only by ER stress.[33] Our study showed that ischaemia/reperfusion significantly stimulated the expression of CHOP (Figure 5), but not caspase-12 activation (data not shown). These results emphasized that ischaemia/reperfusion not only induced the UPR, but if severe, the UPR receptors launched a proapoptotic response that could drive the cell towards programmed death. Considering the postulation that an apoptotic programme is initiated upon severe ER stress, the flavonoid-induced regulatory effect on ER stress signalling may be an important mechanism in its protective effect against cardiac ischaemia/reperfusion.[2,29]

Conclusions

Although this study did not document clearly the specific significance of ER stress protein expression in this system, it suggested that cardiac ischaemia/reperfusion was related to the ER stress response, in which mitochondrial Bcl-2 proteins were also involved. Flavonoids with well-known cardiac protection roles regulate the ER stress response and are probably associated with mitochondrial cell death pathways, leading to cardiac cell protection. This study did not include in-vivo data and the relevance of anti-apoptotic effects of in-vitro ischaemia/reperfusion injury in H9c2 cells may be limited. However, there are several studies about the effect of flavonoids in the model of heart ischaemia.[37–44] Biochanin A, daidzein, genistein, luteolin, quercetin and rutin require further study of their mechanism of ER stress in heart ischaemia or ischaemia/reperfusion *in vivo*.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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