Dual character of flavonoids in attenuating and aggravating ischemia-reperfusion-induced myocardial injury

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Abstract. The concept that flavonoids exert cardioprotection against myocardial ischemia-reperfusion (I/R) injury has been acknowledged by a large body of evidence. However, recent studies reported cardiotoxic effects of certain flavonoids, while the underlying mechanisms have remained largely elusive. Flavonoids have been demonstrated to activate aryl hydrocarbon receptor (Ahr), which is implicated in an array of cell signaling processes. The present study examined the cardioprotective roles of quercetin (Qu) and β-naphthoflavone (β-NF) against I/R injury and explored whether the underlying mechanism proceeds via molecular signaling downstream of Ahr. An oxygen glucose deprivation/reoxygenation (OGD/R) model of I/R was established in myocardial H9c2 cells in the absence or presence of Qu or β-NF. Qu as well as β-NF reversed OGD/R-induced overproduction of reactive oxygen species by increasing the anti-oxidative capacity of the cells and protected them from lethal injury, as demonstrated by a decreased cell death rate, lactate hydrogenase leakage and caspase-3 activity as determined by flow cytometry, colorimetric assay and western blot analysis, respectively. Immunocytochemistry, co-immunoprecipitation and western blot assays collectively revealed that Qu and β-NF engendered the translocation of Ahr from the cytoplasm into the cell nucleus, where binding of Ahr with the Ahr nuclear translocator (ARNT) blocked its binding to hypoxia-inducible factor (HIF)-1α, which inhibited the cardioprotection of HIF-1α, including the induction of nitric oxide (NO) and inhibition of vascular endothelial growth factor (VEGF) production. Ahr knockdown recovered the binding of ARNT to HIF-1α and the generation of NO and VEGF. The results of the present study suggested a dual character of Qu and β-NF in the process of myocardial I/R.

Introduction

Myocardial ischemia induced by acute coronary syndromes remains one of the leading causes of mortality worldwide, as the heart, which has a high energy demand for its normal function, is susceptible to ischemia-induced oxygen and glucose deficiency, which may induce heart damage and even heart failure (1,2). Restoration of blood to the ischemic heart, termed reperfusion, either by thrombolysis or primary percutaneous coronary intervention, is a remedial measure, which facilitates functional rehabilitation of the heart and attenuates cardiac apoptosis and necrosis. However, experimental evidence has largely indicated that timely coronary reperfusion, in turn, paradoxically harms cardiomyocytes. This phenomenon is referred to as myocardial ischemia/reperfusion (I/R) injury, for which particularly the excessive production of reactive oxygen species (ROS) has been held accountable. Thus, current therapeutic guidelines highlight the supplementation with anti-oxidative substances during myocardial I/R (1-3).

Flavonoids are a large group of compounds (n>4,000) that share a common three-ring structure, but have different substituents. Although flavonoids have been identified to have non-caloric and non-nutrient characteristics, numerous members of this compound class exhibit multiple beneficial bioactivities, including anti-oxidant, anti-inflammatory, anti-apoptotic and anticancer properties, and flavonoids are therefore commonly deemed as promising agents for inhibiting ROS-mediated myocardial damage. Over the past few decades, this concept has been strongly supported by numerous studies. Quercetin (Qu), a flavonoid occurring in various vegetables and traditional Chinese herbal medicines, has been shown to attenuate myocardial injury induced by myocardial ischemia-reperfusion (I/R), doxorubicin, and xanthine/xanthine oxidase (X/XO) through neutralizing ROS and regulating an array of signaling molecules (4-8). As a widely distributed flavonoid in fruits, hesperidin has been shown to significantly improve the inotropic and lusitropic function of the heart, as well as to reduce left ventricular end-diastolic pressure, the

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level of thiobarbituric acid reactive substances as a marker of lipid peroxidation and the activity of lactate dehydrogenase as a cardiac injury marker, in an animal model of heart I/R (9). In addition to these flavonoids available from plants, substantial evidence has indicated that β-naphthoflavone (β-NF), an artificially synthesized flavonoid, exerts anti-oxidative action via stimulating the activities of diverse anti-oxidant enzymes and averting doxorubicin-induced myocardial damage (10-14). However, certain recent studies have reported cardiotoxic activities of flavonoids (e.g. Qu and β-NF) (13,14), although the underlying mechanisms have remained to be largely elucidated.

Flavonoids have been reported to act as agonists of the aryl hydrocarbon receptor (Ahr) (15). Ahr is a ligand-activated transcription factor, which is resident in the cytoplasm in its latent form, bound to heat shock protein 90 (HSP90). Upon ligand-mediated activation, Ahr rapidly translocates to the nucleus, where it dissociates from HSP90 and heterodimerizes with the Ahr nuclear translocator (ARNT). The Ahr/ARNT complex then binds to specific recognition sites and initiates the transcription of target genes of Ahr (16,17). However, the transcriptional activation regulated by hypoxia-inducible factor (HIF)-1α also relies on the formation of a complex with ARNT. HIF-1α is an oxygen-sensitive transcription factor, which exerts a vast array of physiological functions, enabling cells to adapt to temporary hypoxia (18,19). Studies have reported that HIF-1α becomes highly labile under hypoxic conditions induced by myocardial ischemia and it is allowed to translocate to the nucleus to trigger transcriptional activation of genes associated with cardioprotection, such as vascular endothelial growth factor (VEGF), inducible nitric oxide (NO) synthase (iNOS), erythropoietin and heme oxygenase-1 (18,19). Based on these findings, the present study speculated that Ahr activated by certain flavonoids competes with HIF-1α for binding to ARNT, resulting in the inhibition of cardioprotection mediated by HIF-1α, which may limit or counteract the beneficial actions of flavonoids against myocardial I/R.

H9c2 cells initially derived from mitotic rat embryonic cardiomyocytes have been widely used as a model system to study cardioprotective and cardiotoxic effects of various substances in various settings. The present study established a cellular oxygen glucose deprivation/reoxygenation (OGD/R) model of myocardial I/R using H9c2 cells and further examined the cardioprotective roles of Qu and β-NF under these conditions. Further research efforts were devoted to exploring whether the interaction between Ahr and HIF-1α mediates the detrimental effects of Qu and β-NF in myocardial OGD/R.

Materials and methods

Cell culture and treatments. H9c2 cells used in the present study were purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in a 37°C incubator with 5% CO_2. After reaching 70-80% confluence, the cells were subjected to oxygen glucose deprivation/reoxygenation (OGD/R) to simulate I/R injury, as described in the study of Zhao *et al* (2), in the absence or presence of Qu or β-NF. In brief, cells were exposed to hypoxic conditions (oxygen deprivation, 0.5% O_2) for 24 h in culture medium deprived of glucose and serum, followed by culture under normoxic conditions (reoxygenation) in normal medium for an additional 24 h. Qu (0.5, 5 and 50 μM) and β-NF (0.1, 1 and 10 μM; Sigma-Aldrich; Merck KGaA, Darmstadt Germany) were individually added to the cells during the entire process of OGD/R.

Immunocytochemical (ICC) assay. An ICC assay was used to analyze the protein levels of Ahr in the nuclei of H9c2 cells subjected to the abovementioned treatments. The cells were fixed with 4% paraformaldehyde for 15 min and blocked with PBS containing 0.3% Triton X-100 and 5% bovine serum albumin (w/v) (Gibco; Thermo Fisher Scientific, Inc.) for 1 h at room temperature. Subsequently, the cells were incubated with primary antibody specific for Ahr (1:500 dilution; cat. no. ab2770; Abcam, Cambridge, UK) for 2 h at room temperature prior to incubation with the secondary fluorescent-labeled antibody (A-21202; Alexa Fluor 488; Invitrogen; Thermo Fisher Scientific, Inc.) at room temperature for 1 h. DAPI (1:1,000 dilution; Invitrogen; Thermo Fisher Scientific, Inc.) and fluorescence-quenching agent (TF-B21; Weifang Greatland Chemicals Co., Ltd, Shandong, China) were added to the cells in the dark for 5 min, followed by analysis with a fluorescence microscope (Nikon, Tokyo, Japan) and analysis of the images using DP2-BSW software (Olympus, Tokyo, Japan).

Knockdown of Ahr in H9c2 cells. Small interfering RNA (siRNA) targeting Ahr (siRNA-Ahr) was synthesized by GenePharma Co., Ltd. (Shanghai, China). siRNA-Ahr was transfected into H9c2 cells using Lipofectamine™ 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. H9c2 cells with Ahr knockdown were further subjected to OGD/R in the absence or presence of Qu or β-NF.

Determination of apoptotic rate. The apoptotic rate of H9c2 cells was assessed by using an Annexin V-fluorescein isothiocyanate/propidium iodide kit (Kaiji Biological Inc., Nanjing, China) according to the manufacturer's protocol. A dual laser flow cytometer (Becton Dickinson, San Jose, CA, USA) with ModFit LT software (Verity Software House, Topsham, ME, USA) was used to determine the apoptotic rate.

Lactate hydrogenase (LDH) leakage assay. The amount of LDH in the culture medium was examined with using a LDH Activity Assay kit (Beyotime Institute of Biotechnology, Haimen, China). After the respective treatments, cell culture medium was collected and transferred to a 96-well plate. LDH reaction mix was added to each well, and the plates were incubated for 30 min at room temperature. Finally, the optical density was determined at 450 nm using an ELISA plate reader (Model 550; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Measurement of intracellular ROS and cell total anti-oxidant capacity (TAOC). Intracellular ROS levels in H9c2 cells were quantified using a Reactive Oxygen Species Assay kit (Beyotime Institute of Biotechnology). After washing with

PBS, the cells were suspended in 2',7'-dichlorofluorescin diacetate (DCFH-DA) solution (10 μM) at 10^7/ml and incubated at 37°C for 20 min. The fluorescence intensity of DCFH-DA in the cells was detected by a fluorospectrophotometer (F-4000; Hitachi, Ltd., Tokyo, Japan).

The azino-diethyl-benzthiazoline sulfate (ABTS) method was adopted to examine the TAOC of H9c2 cells. Incubation with ABTS with H_2O_2 and a peroxidase (metmyoglobin) (provided by ABTS detection kit from Beyotime Institute of Biotechnology) results in the production of a blue-green radical cation ABTS$^+$. Anti-oxidants contained in the H9c2 cells suppress this color production proportionally to the cells' TAOC. The system was standardized using Trolox (provided by ABTS detection kit), a water-soluble vitamin E analogue. The results were expressed as μmol Trolox equivalent/protein concentration of the H9c2 cells.

Intracellular NO measurement. NO in the H9c2 cells was detected using the NO Detection kit (Beyotime Institute of Biotechnology) according to the manufacturer's instructions. In brief, the level of the NO derivative nitrite was determined via the Griess reaction. A standard curve was generated using $NaNO_2$ mixed with Griess reagent. After 15 min, optical density was read using a microplate reader at 540 nm.

Western blot analysis. Total proteins were extracted from H9c2 cells with a cell lysis reagent (Sigma-Aldrich; Merck KGaA) according to the manufacturer's instructions and protein was quantified using the bicinchoninic acid method (Beyotime Institute of Biotechnology). The extracted proteins (20 μg per lane) were separated by 10-15% SDS-PAGE and transferred onto nitrocellulose membranes (EMD Millipore, Billerica, MA, USA). Membranes were blocked in 5% non-fat milk in Tris-buffered saline/0.1% Tween-20 for 2 h prior to immunoblotting at 4°C overnight with the following antibodies: Anti-Ahr, anti-HIF-1α (1:300 dilution, cat. no. ab463; Abcam), anti-Caspase-3 (1:500 dilution, cat. no. ab90437; Abcam), iNOS (1:500 dilution, cat. no. ab15323; Abcam), VEGF (1:800 dilution, cat. no. bs-1665R; Bioss, Beijing, China) and GAPDH (1:1,000 dilution, cat. no. sc-365062; Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Membranes were then incubated with horseradish peroxidase-conjugated secondary antibody (1:20,000 dilution; cat. no. A9309, Sigma-Aldrich, Merck KGaA; and cat. no. ab97051, Abcam) for 2 h at room temperature. The intensities of the bands were quantified using an enhanced chemiluminesence detection kit (Pierce; Thermo Fisher Scientific, Inc.) and an image analysis system (ProteinSimple; Bio-Techne, Minneapolis, MN, USA).

Co-immunoprecipitation (Co-IP) assay. Nucleoprotein in H9c2 cells was extracted with a CelLytic™ NuCLEAR™ Extraction kit (Sigma-Aldrich; Merck KGaA) and incubated ARNT primary antibody (1:500 dilution, cat. no. sc-5580; Santa Cruz Biotechnology, Inc.) at 4°C for 60 min with gentle mixing. Subsequently, 20 μl Protein A/G Plus-Agarose beads (Thermo Fisher Scientific, Inc.) was added, followed by incubation at 4°C overnight. The mixture was centrifuged at 500 x g for 5 min at 4°C. The supernatant was discarded and the Co-IP products were washed three times with PBS. After the final wash, the precipitates were re-suspended in 40 μl

sample buffer and detected by western blotting with anti-Ahr (1:200 dilution) and anti-HIF-1α antibodies (1:200 dilution).

Statistical analysis. Values are expressed as the mean ± standard deviation. Statistical analysis was performed using SPSS v12.0 software (SPSS, Inc., Chicago, IL, USA). One-way analysis of variance with Scheffe's post-hoc testing was used for multiple comparisons between two groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Qu and β-NF drive the translocation of Ahr from cytoplasm to cell nucleus. Ahr is a receptor mainly present in the cytoplasm in its latent form, while it translocates from the cytoplasm into the cell nucleus to trigger target gene expression once activated by its ligands. An immunocytochemical assay demonstrated that H9c2 cells subjected OGD/R alone showed no significant change in Ahr fluorescence intensity (FI) in the cell nucleus (Fig. 1). Supplementation with Qu at 5 or 50 μM during the OGD/R process increased Ahr FI in the cell nucleus (P<0.05 vs. control group). Furthermore, Ahr FI in the cell nucleus was dose-dependently increased by β-NF within the tested concentrations in the OGD/R process, with 10 μM β-NF causing the greatest increase (P<0.05 vs. control group). Based on these findings, 5 μM Qu and 10 μM β-NF were selected as the treatment concentrations used in subsequent assays.

Qu and β-NF inhibit cell death, LDH leakage and caspase-3 activation caused by OGD/R. Exposure of H9c2 cells to OGD/R induced an increase in the apoptotic rate (P<0.05 vs. control group, Fig. 2A), which was individually decreased by 5 μM Qu and 10 μM β-NF (P<0.05 vs. OGD/R group). Ahr knockdown in H9c2 cells attenuated the protective effect of 10 μM β-NF against cell death (P<0.05), but barely influenced the protective effect of 5 μM Qu. LDH leakage is an important indicator of myocardial insult induced by I/R. Treatment with OGD/R augmented LDH leakage from H9c2 cells (P<0.01 vs. control group; Fig. 2B), whereas this action was individually inhibited by 5 μM Qu and 10 μM β-NF (P<0.05 vs. OGD/R group). The protective effect of 5 μM Qu and 10 μM β-NF against LDH leakage was diminished after Ahr knockdown (P<0.05). As a critical apoptotic marker, caspase-3 activity is positively correlated with the cleaved caspase-3 protein level. An increase in the levels of cleaved caspase-3 protein in H9c2 cells was observed after OGD/R compared with that in the control group (P<0.05, Fig. 2C). Supplementation with 5 μM Qu during the OGD/R process inhibited the increase of cleaved caspase-3 in H9c2 cells (P<0.05 vs. OGD/R group). Ahr knockdown did not influence the inhibitory effect. Addition of 10 μM β-NF to H9c2 the cells diminished the upregulation of cleaved caspase-3 induced by OGD/R (P<0.05 vs. OGD/R group). However, Ahr knockdown attenuated the inhibition of cleaved caspase-3 by β-NF (P<0.05 vs. β-NF group without knockdown).

Qu and β-NF inhibit intracellular ROS production and decreases in cell TAOC during OGD/R. H9c2 cells subjected to OGD/R showed increased intracellular ROS levels compared with those in the control (P<0.01; Fig. 2D). Supplementation with 5 μM Qu during the OGD/R process lowered the ROS
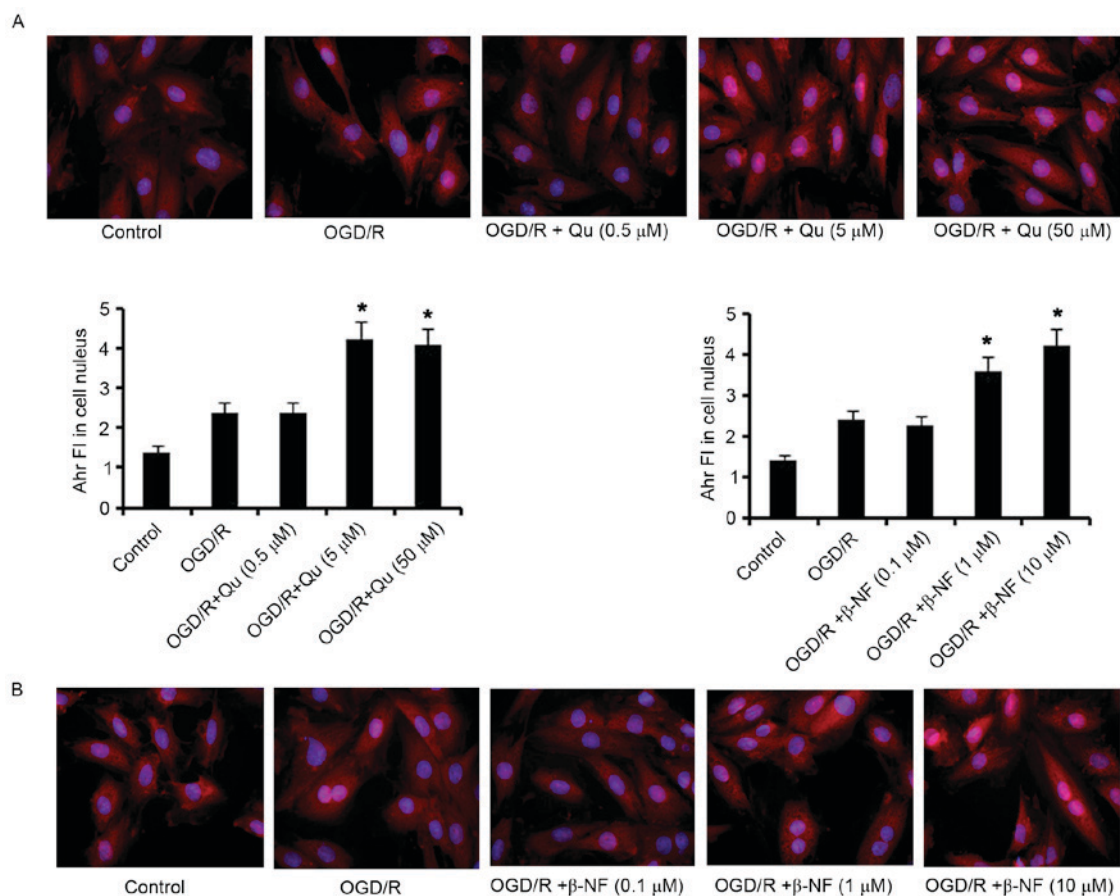
Figure 1. Qu and β-NF lead to the translocation of Ahr from cytoplasm to cell nucleus. H9c2 cells were exposed to hypoxic conditions (oxygen deprivation, 0.5% O₂) for 24 h in culture medium deprived of glucose and serum, followed by culture under normoxic conditions (reoxygenation) in normal medium for an additional 24 h. (A) Qu (0.5, 5 and 50 µM) and (B) β-NF (0.1, 1 and 10 µM) were added individually to the cells during the whole process of OGD/R. An immunocytochemical assay was used to analyze the protein level of Ahr in the nuclei in treated H9c2 cells. Magnification, x400. *P<0.05 vs. control. Ahr, aryl hydrocarbon receptor; Qu, quercetin; β-NF, β-naphthoflavone; OGD/R, oxygen glucose deprivation/reoxygenation; FI, fluorescence intensity.

in H9c2 cells (P<0.01 vs. OGD/R group), but Ahr knockdown partly attenuated the anti-oxidative function (P<0.05 vs. Qu group without knockdown). Decreased ROS were also observed in H9c2 cells when 10 µM β-NF was added during the OGD/R process (P<0.05 vs. OGD/R group). However, Ahr knockdown reversed the anti-oxidative function (P<0.05 vs. β-NF group without knockdown). OGD/R decreased the cell TAOC relative to that in the control (P<0.01), which was attenuated by 5 µM Qu and 10 µM β-NF (P<0.05 vs. OGD/R group; Fig. 2E). Ahr knockdown in H9c2 cells inhibited the effect of Qu and β-NF on increasing TAOC (both P<0.05).

NO content in H9c2 cells after various treatments. The NO content in H9c2 cells was decreased after H9c2 cells were subjected to OGD/R compared with that in the control cells (P<0.05; Fig. 2F). Supplementation with 5 µM Qu or 10 µM β-NF in the OGD/R process resulted in a further decrease in NO content compared with that in the OGD/R-treated group (P<0.05). Ahr knockdown conversely increased NO content in H9c2 cells that were subjected to OGD/R in the presence of Qu and β-NF, compared with the cells without Ahr knockdown (both P<0.01).

Target protein levels after various treatments. Western blot assay revealed that HIF-1α protein levels were upregulated

after H9c2 cells were subjected to OGD/R (P<0.05 vs. control; Fig. 3A). In comparison to the OGD/R-treated group, supplementation with 5 µM Qu or 10 µM β-NF in the process of OGD/R did not significantly affect HIF-1α protein levels regardless of whether Ahr knockdown was performed. iNOS protein levels in H9c2 cells were significantly decreased by treatment with OGD/R (P<0.05). Addition of 5 µM Qu or 10 µM β-NF to cells in the process of OGD/R further decreased iNOS protein levels (P<0.05 vs. OGD/R group), which was reversed by Ahr knockdown (P<0.01 vs. the Qu and β-NF groups without knockdown). OGD/R treatment promoted VEGF protein expression in H9c2 cells (P<0.05 vs. control group), which was significantly inhibited by supplementation with 5 µM Qu or 10 µM β-NF (P<0.05 vs. OGD/R group). However, Ahr knockdown impaired the inhibited effect of Qu and β-NF on VEGF expression (P<0.05 and P<0.01, respectively).

Ahr competes with HIF-1α for combining with ARNT after stimulation with Qu and β-NF. The Co-IP assay indicated that OGD/R promoted the binding of ARNT to HIF-1α (P<0.05 vs. control group, Fig. 3B), but had little effect on the binding of ARNT to Ahr. Supplementation with Qu and β-NF during OGD/R promoted the binding of ARNT to Ahr (both P<0.05 vs. OGD/R group), but decreased the binding of
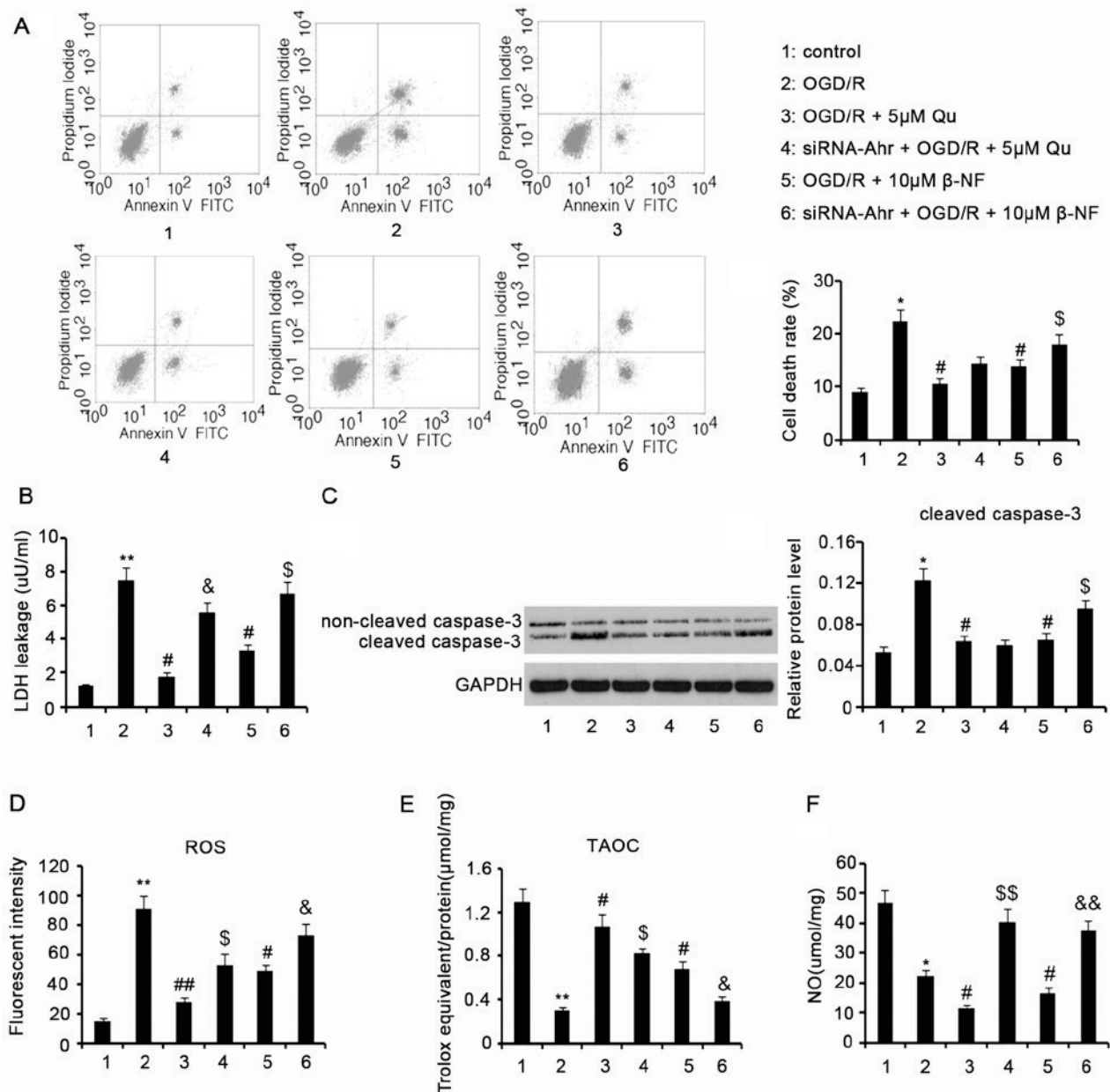
Figure. 2 Qu and β-NF protect cells from lethal injury and decrease ROS in cells. H9c2 cells with or without Ahr knockdown were exposed to hypoxic conditions (oxygen deprivation, 0.5% O₂) for 24 h in culture medium deprived of glucose and serum, followed by culture under normoxic conditions (reoxygenation) in normal medium for an additional 24 h. 5 μM Qu or 10 μM β-NF was added individually to the cells during the entire process of OGD/R. (A) The cell death rate was assessed by flow cytometry. The total number of cells in each group was 2×10^7. (B) Lactate hydrogenase leakage was assessed by a colorimetric assay. (C) Cleaved caspase-3 protein levels were assessed by western blot analysis. (D) Intracellular ROS, (E) TAOC of the cells and (F) NO levels were assessed after the treatments. Groups: 1, Control; 2, OGD/R; 3, OGD/R + 5 μM Qu; 4, siRNA-Ahr + OGD/R + 5 μM Qu; 5, OGD/R + 10 μM β-NF; 6, siRNA-Ahr + OGD/R + 10 μM β-NF. *P<0.05, **P<0.01 vs. control group; #P<0.05, ##P<0.01 vs. OGD/R group; $P<0.05 and $$P<0.01 vs. Qu group without Ahr knockdown; &P<0.05 and &&P<0.01 vs. β-NF group without Ahr knockdown. ROS, reactive oxygen species; Qu, quercetin; β-NF, β-naphthoflavone; OGD/R, oxygen glucose deprivation/reoxygenation; Ahr, aryl hydrocarbon receptor; siRNA-Ahr, small interfering RNA against Ahr; FITC, fluorescein isothiocyanate; TAOC, total antioxidant capacity; NO, nitric oxide.

ARNT to HIF-1α (both P<0.05 vs. OGD/R group). However, knockdown of Ahr in H9c2 led to a decrease in the binding of ARNT to Ahr (P<0.01 vs. Qu and β-NF groups without Ahr knockdown) and an increase in the binding of ARNT to HIF-1α (P<0.01 vs. Qu group without Ahr knockdown and P<0.05 vs. β-NF group without Ahr knockdown) in the presence of Qu and β-NF. These data in combination with results from the ICC assay indicate that Qu and β-NF promote nuclear translocation of Ahr; Ahr binds to ARNT

in the nucleus, resulting in reduced binding of ARNT to HIF-1α.

Discussion

Previous studies have extensively reported that ROS are largely produced in myocardial ischemia as well as reperfusion, although the underlying mechanisms are probably different (3). Continuous deficiency of oxygen and glucose
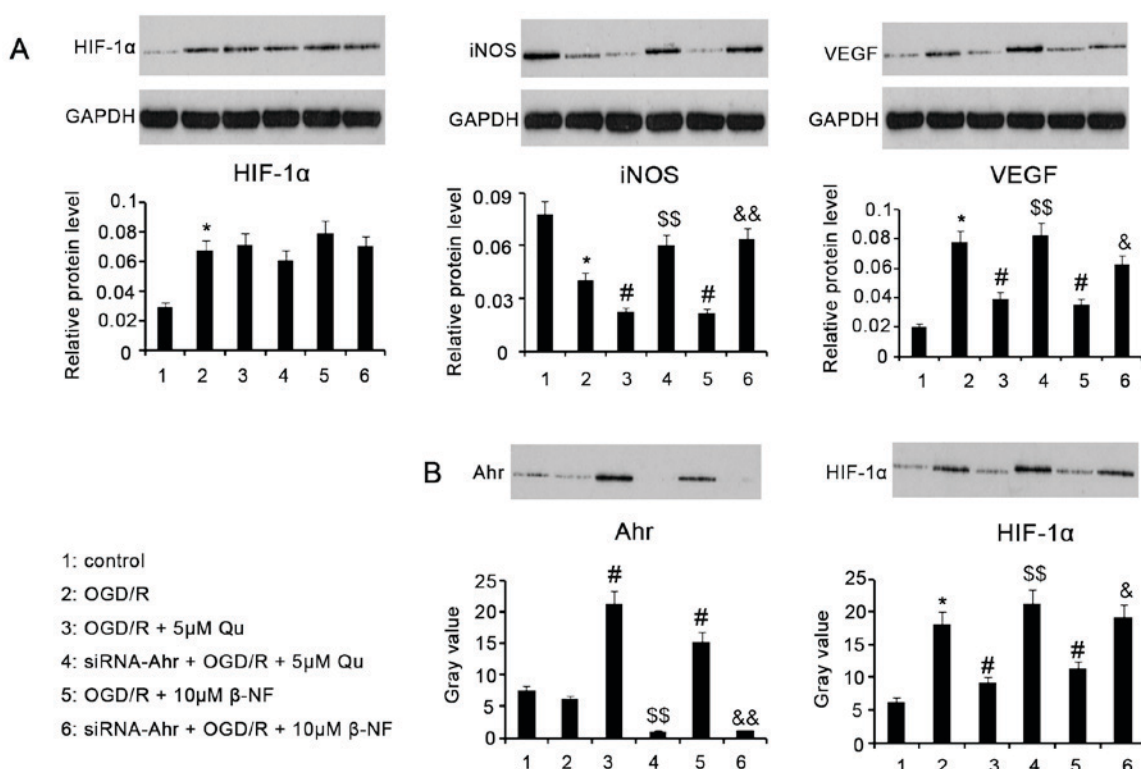
Figure 3. Target protein levels after various treatments. H9c2 cells with or without Ahr knockdown were exposed to hypoxic conditions (oxygen deprivation, 0.5% O$_2$) for 24 h in culture medium deprived of glucose and serum, followed by culture under normoxic conditions (reoxygenation) in normal medium for an additional 24 h. 5 μM Qu and 10 μM β-NF were added individually to the cells during the whole process of OGD/R. (A) Western blot analysis was used for assessing the levels of HIF-1α, iNOS and VEGF in the treated cells. (B) A co-immunoprecipitation assay was used to test the interaction between Ahr and HIF-1α in terms of ARNT binding in the nuclei of treated cells. Nucleoprotein extracted from H9c2 cells was incubated with ARNT primary antibody and then subjected to western blotting with anti-Ahr or anti-HIF-1α antibodies. Groups: 1, Control; 2, OGD/R; 3, OGD/R + 5 μM Qu; 4, siRNA-Ahr + OGD/R + 5 μM Qu; 5, OGD/R + 10 μM β-NF; 6, siRNA-Ahr + OGD/R + 10 μM β-NF. *P<0.05 vs. control group; #P<0.05 vs. OGD/R group; $P<0.05, $$P<0.01 vs. Qu group without Ahr knockdown; &P<0.05, &&P<0.01 vs. β-NF group without Ahr knockdown. Qu, quercetin; β-NF, β-naphthoflavone; OGD/R, oxygen glucose deprivation/reoxygenation; Ahr, aryl hydrocarbon receptor; siRNA-Ahr, small interfering RNA against Ahr; ARNT, Ahr nuclear translocator; iNOS, inducible nitric oxide synthase; HIF, hypoxia-inducible factor; VEGF, vascular endothelial growth factor.

during myocardial ischemia disrupts mitochondrial homeostasis and metabolism, facilitating the conversion of O$_2$ to O$_2^-$ and other ROS due to increased electron leakage. Timely reperfusion indeed eases ischemic injury and salvages viable myocardium, while NADPH oxidases, lipoxygenase and xanthine oxidase are activated in response to the reperfusion, which are responsible for the generation of most of the ROS in this process. The present study established a cellular OGD/R model of I/R and showed that ROS was markedly and consistently elevated in these treated H9c2 cells (3). The significantly increased ROS leads to a huge consumption of anti-oxidative substances and suppresses the activities of certain anti-oxidant enzymes, resulting in an attenuated cell TOAC. In addition to ATP depletion and Ca^{2+} overload, the disruption of the oxidative and anti-oxidative balance has in OGD/R been associated with myocardial damage and death, (3). In agreement with previous studies, the results of the present study showed that the apoptotic rate, LDH leakage and caspase-3 activity were increased in parallel with the significantly elevated ROS.

The anti-oxidative properties of Qu and β-NF deserve to be acknowledged, based on the results of the present and previous studies. In the present study, supplementation with

Qu or β-NF during the OGD/R process notably diminished ROS in H9c2 cells and reinforced the cell TOAC. Several studies have demonstrated that Qu has the capability to scavenge superoxide anions, singlet oxygen and lipid peroxy radicals *in vitro* and in animal models (4-8). β-NF has been shown to strengthen the activities of anti-oxidative enzymes, such as glutathione peroxidase, quinone oxidoreductase 1, glutathione transferase and heme oxygenase 1, and to repress NADPH oxidases that are ROS-producing enzymes, thereby having an important anti-oxidant role (10-14). Qu and β-NF caused decreases in ROS in H9c2 cells, thus protecting the cells from death and impairment resulting from exposure to OGD/R. An accidental discovery of the present study was that Ahr knockdown notably attenuated the anti-oxidative action of β-NF, suggesting that Ahr mediated the anti-oxidative action. Slightly different from β-NF, the anti-oxidative capacity of Qu was partly decreased by Ahr knockdown, which suggested that anti-oxidative function of Qu is at least partly dependent on Ahr signaling.

The cardioprotective actions mediated by HIF-1α in myocardial I/R have been partly elucidated. The stability of HIF-1α is regulated by the HIF-prolyl hydroxylases domain (PHD) that targets it for polyubiquitination and proteosomal

degradation. The hypoxia induced by myocardial ischemia inhibits the activity of HIF-PHD, thereby allowing HIF-1α to accumulate and translocate to the nucleus, where it binds to ARNT and regulates the transcription of certain hypoxia-responsive genes (20-22). Certain experimental studies have shown that genetic or pharmacological stabilization of HIF-1α protects the heart against the detrimental effects of acute I/R injury by enhancing iNOS, VEGF and B-cell lymphoma-2 expression and restricting nuclear factor-κB-dependent gene expression (20-22). In the present study, HIF-1α protein levels in H9c2 cells were markedly elevated in response to OGD/R, which represents a self-protective mechanism of myocardial H9c2 cells in response to this challenge. Although no significant difference in HIF-1α protein levels was observed with Qu and β-NF addition during OGD/R, the ICC and Co-IP assays revealed that Qu and β-NF promoted the translocation of Ahr from the cytoplasm into the cell nucleus, where Ahr dimerized with ARNT to significantly decrease the amount of ARNT that binds to HIF-1α. Ahr is a cytosolic ligand-activated transcription factor that can be activated by a class of flavonoids. Activated Ahr translocates into the cell nucleus and binds to ARNT to form the Ahr/ARNT complex, and then binds to specific recognition sites in its target genes. As a likely consequence of ARNT binding to Ahr present in large amounts but rarely to HIF-1α, the cardioprotection mediated by HIF-1α is attenuated.

In accordance with this presumption, iNOS and VEGF protein levels together with the NO content were significantly decreased in H9c2 cells after treatment with Qu or β-NF. Knockdown of Ahr in H9c2 cells in the presence of Qu or β-NF increased binding of ARNT to HIF-1α, which was accompanied with marked increases in iNOS and VEGF protein levels as well as NO formation. It has been well-documented that NO exerts robust cardioprotective effects against I/R injury. NO is a gaseous signaling molecule that participates in a wide variety of cardiovascular functions, including vasodilatation, neovascularization, scavenging of ROS and regulation of the cardiac immune response. Studies have revealed a reduction in the bioavailability of NO in the reperfusion phase, while interventions that can increase NO formation have a significant therapeutic effect against myocardial I/R injury (23-25). The importance of VEGF in the inhibition of the progression of myocardial I/R injury has been recognized for several years. Several substances showing protection against myocardial I/R injury have been demonstrated to act in a VEGF-dependent manner (26). VEGF gene therapy for the purpose of promoting therapeutic angiogenesis and recovering the pressure developed in the left ventricle has now been advanced as an alternative treatment for myocardial I/R (22,26). It is therefore likely that flavonoid-induced decreases in NO and VEGF formation probably exacerbate myocardial I/R injury.

In conclusion, the present study confirmed the anti-oxidative and protective effects of Qu and β-NF in the OGD/R process of H9c2 cells. More importantly, the present study revealed that Qu and β-NF attenuated the HIF-1α-mediated cardioprotection through activating Ahr. The results provided by the present study suggest a dual character of certain flavonoids in the process of myocardial I/R.

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