RETRACTED ARTICLE: Quercetin supports cell viability and inhibits apoptosis in cardiocytes by down-regulating miR-199a

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ABSTRACT

Hypoxia-caused cardiocytes insults are closely correlated with ectopic expression of genes, which might be modulated by microRNAs (miRs). Quercetin exhibits a profound protective function against hypoxic damages in cardiomyocytes. Here, we aimed to investigate a possible underpinning. H9c2 cells were pre-administrated using quercetin before hypoxia treatment. The damages were assessed using viability, apoptosis and alteration of proteins associated with apoptosis and adenosine monophosphate-activated protein (AMPK) pathway. Transfection was conducted to enforce overexpression of miR-199a or silence of sirtuin 1 (sirt1) which were confirmed by qRT-PCR. Sirt1 protein was quantified by immunoblotting. A luciferase reporter was exploited to confirm the target relationship between miR-199a and sirt1 3'-untranslated region (3'-UTR). We found quercetin mitigated hypoxia-caused viability reduction and apoptosis with restoring apoptosis-associated protein and rescuing phosphorylation of AMPK. Quercetin flattened hypoxia-evoked overexpression of miR-199a. miR-199a abrogated the protective effects of quercetin against hypoxia-elicited damages. Quercetin elevated sirt1 which was repressed by hypoxia, while this effect was slight in miR-199a-overexpressed cells. miR-199a negatively mediated sirt1 expression through directly binding its 3'-UTR. Further, quercetin facilitated the phosphorylation of AMPK by up-regulating sirt1. Collectively, quercetin participated in repressing miR-199a which negatively modulated sirt1. Mechanically, through activating AMPK, quercetin protected cardiomyocytes cells against hypoxia-caused insults.

HIGHLIGHTS

- Quercetin ameliorates hypoxia-evoked apoptosis and blockage of AMPK phosphorylation;
- The elevated miR-199a level is eased by quercetin, which might be a protective mechanism;
- Quercetin restores sirt1 level by repressing miR-199a expression;
- By mediating miR-199a and sirt1, AMPK phosphorylation is fortified by quercetin.

Introduction

Cardiovascular disease (CVD) is responsible for 17.0 million deaths each year, accounting for 31% of all deaths worldwide and even estimated to cause 25 million deaths by 2030 [1]. Considerable results suggest that oxidative stress emerges as a pivotal feature of CVD, and hypoxia condition induces oxidative stress [2]. Mostly, hypoxia is detrimental to the functionality of hearts as cardiomyocytes experience apoptosis in response to this stimulus, which might evolve as a loss of function [3]. The interrupt of cardiac function causes critical and permanent damage to the whole human body. Because cardiomyocytes deletion is the critical determinant of morbidity and mortality, blocking apoptosis progress is regarded as an effective preventive or therapeutic strategy for CVD.

Overwhelming evidence derived from human epidemiology studies proves that hypoxic condition increases susceptibility to CVD. Hypoxia-elicited accumulation of gene expression changes results in pathological reactive fibrosis [4]. Of particular importance, microRNAs (miRs) are identified as crucial mediators of gene expression, which has been observed to be associated with a physiological outcome [5]. miR-199a is tissue-specifically expressed in cardiomyocytes and its up-regulation is linked with hypertrophy [6]. Most recently, a report revealed that miR-199a-214 cluster exerts a function on a metabolic shift from predominant reliance on fatty acid utilization toward increased reliance on glucose metabolism at the onset of heart failure [7]. Moreover, miR-199a has been reported to be involved in cardiac hypoxic damage by targeting sirtuin 1 (sirt1) [8], which mediates apoptosis in cardiac myoblasts in response to hypoxia [9].
Antioxidant therapeutic protection against CVD has been proposed. A growing body of natural products from dietary intake has been shown to harbor health benefits against CVD induced by oxidative stress [10,11]. Quercetin is widely categorized as a flavonol and daily consumed from plant-derived food. Its possible health-beneficial effects have been elucidated to be associated with its ability to quench free radicals [12]. Its therapeutic potential and molecular underpinnings as a cardiovascular agent have been discussed in the improvement of heart features and treatment of cardiac diseases [13]. Considerably, quercetin exhibits its biological properties via regulating miRs and sirt1 network in apoptosis processes [14]. However, there exist no reports on cardio-protection of quercetin against hypoxia via regulating miR-199a-sirt1 axis.

In order to explore the potency of quercetin as a cardiovascular agent, rat-derived cardiomyocytes H9c2 cells were exploited to establish cell damage model by culturing in hypoxia environment. Furthermore, in the present investigation, we evaluated the protective mechanism of quercetin, which might be ascribed to the miR-199a-sirt1 axis as shown in Figure 1.

Materials and methods

Cell culture and administration

Myocardium myoblast cell line H9c2 cells were purchased from American Type Culture Collection (ATCC) (Rockville, MD, USA). According to general information from the supplier, these cells were derived from Rattus norvegicus, rat. Consistent with suggested culture method, the cells were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) (Gibco, Gaithersburg, MD, USA) adding with fetal bovine serum (Gibco) at a final concentration of 10% (v/v). The culture was maintained in a humid atmosphere with 5% CO2 and 95% air at 37°C. Quercetin was commercial production obtained from Sigma-Aldrich (St. Louis, MO, USA). Its purity was more than 95% tested by high-pressure liquid chromatography. Quercetin was diluted in dimethyl sulfoxide (DMSO) (Sigma-Aldrich) at a final concentration of 100 mM for storage. Then, the stored solution was attenuated by DMEM into different concentrations (0–50 μM). H9c2 cells were pre-treated by quercetin for 24 h with a control group, which was treated by an equal amount of DMSO. As for hypoxia treatment, the cells were subsequently incubated in a hypoxic incubator containing 94% N2, 5% CO2 and 1% O2 for 12 h.

Transfection

GMR-miRTM microRNA-199a single-stranded mimics are exogenously synthesized by GenePharma (Shanghai, China), which could imitate endogenous miR-199a. To enforce H9c2 cells to overexpress miR-199a, we transduced miR-199a mimic and its negative control (NC) into cells. Transduction was performed using Lipofectamine 3000 transfection reagent (Invitrogen, Carlsbad, CA, USA) and transfection efficiency was identified by qRT-PCR. Small interfering RNA (siRNA) was applied to silence sirt1 at mRNA level, therefore, as well as protein level. Sirt1 siRNA oligos (si-sirt1) were purchased from GenePharma and transfected into H9c2 cells with negative control (si-NC). Its transfection efficiency was tested by qRT-PCR and immunoblotting assay.

Cell viability assay

Cell viability was assessed by a commercial kit. Cell counting kit-8 (CCK-8) was purchased from APEXlBO (Houston, TX, USA) containing 2-(2-methoxy-4-nitrophenyl)-3-(4-nitropheryl)-5-(2,4-disulphophenyl)-2H-tetrazolium monosodium salt (WST-8), which can be reduced by dehydrogenases in the presence of an electron mediator. Briefly, 100 μL cell suspension (5000 cells per well) was dispensed in 96-well plate and pre-incubated in a humidified atmosphere at 37°C. The cells were incubated with quercetin and then stimulated by hypoxia in consistent with abovementioned concentrations and time. Next, the cells were co-incubated with 10 μL CCK-8 solution for 1 h. Finally, the absorbance was detected at 450 nm using a Multi-Mode Microplate Reader (Molecular devices, San Jose, CA, USA).

Apoptosis assay

Apoptotic progression was performed using the Annexin V-FITC Apoptosis Detection Kit (Abcam, Cambridge, MA, USA). In short, the cells were collected by centrifugation, gently trypsinized by trypsin (Pierce, Appleton, WI, USA) and washed using serum-containing media. Then, the cells were re-suspended by 500 μL binding buffer. Next, 5 μL Annexin V-FITC and 5 μL propidium iodide was added into cell suspension, which was further incubated for 5 min in the dark at room temperature. Finally, the cells were visualized by CytoFLEX Flow Cytometer (Beckman Coulter, IN, USA).

Figure 1. A proposed axis of miR-199a-mediated anti-apoptotic activity of quercetin in myocardial cells. Quercetin alleviated hypoxia-elicited overexpression of miR-199a, which caused the rescue of sirt1 level resulting in activation of AMPK pathway, and finally ameliorated apoptosis. QUER, quercetin; miR-199a, microRNA-199a; sirt1, sirtuin 1; AMPK, adenosine monophosphate activated protein.
Dual luciferase reporter assay
To confirm miR-199a targeted 3'-UTR of sirt1, we constructed the fragment of sirt1 3'-UTR, which contains the predicted miR-199a binding sites into pGL14 Luciferase reporter vector (Promega, Madison, WI, USA). Site-directed mutagenesis in the seed sequence of miR-199a binding site was introduced using In-Fusion cloning kit (Clontech, Mountain View, CA, USA) as per manufacturer’s protocol. Dual-Luciferase Reporter Assay System was applied for luciferase reporter assay (Promega). HEK 293 T/17 cells (ATCC) was simultaneously transfected with wild type (wt) or mutant (mut) type 3'-UTR sirt1 and miR-199a mimic or NC mimic. Luciferase activity was detected using CytoFLEX Flow Cytometer (Beckman Coulter). Firefly luciferase activity was normalized to Renilla luciferase activity. The radio of sirt1 3'-UTR to NC mimic was set as 1; therefore, the results were suggested as the fold changes relative to control.

Quantitative reverse transcription-PCR (qRT-PCR)
Whole RNA was extracted from H9c2 cells using Trizol reagent (Invitrogen) as described in the manufacturer’s instructions. For miR-199a, RT was conducted using the Taqman MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA). The quantitative PCR was performed using Taqman Universal Master Mix II (Thermo Fisher Scientific). U6 was used as an internal control. As for sirt1, qRT-PCR was conducted using a high capacity cDNA RT Kit (Applied Biosystems, Foster City, CA, USA) and Maxima SYBR Green qPCR Master Mix (Thermo Fisher Scientific). Its relative expression was calculated by the 2^ΔΔCt method relative to β-actin.

Immunoblotting assay
Whole cell lysis was obtained using RIPA lysis buffer (Beyotime, Shanghai, China) and protease inhibitors (Roche Applied Science, Indianapolis, USA) according to user’s protocols. Protein concentration was determined using BCA™ protein assay kit (Pierce). Next, the proteins were separated on a Bio-Rad Bis-Tris Gel system with reference to manufacturer’s description (Bio-Rad, Hercules, CA, USA) and moved onto polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). Blots were probed with primary antibodies against p53, caspase-3, sirt1, β-actin, total (t)-adenosine monophosphate activated protein (AMPK), phosphor (p)-AMPK (Cell Signaling, Hercules, CA, USA), and Bcl-2 (Abcam), diluted in 5% blocking buffer at a dilution of 1:1000 overnight at 4 °C, followed by incubation with goat anti-rabbit antibodies conjugated by horseradish peroxidase (HRP) (Cell Signalling) for 1 h at room temperature. Signals were visualized using Immobilon Western Chemiluminescent HRP substrate (Millipore) on Bio-Rad ChemiDoc™ XRS system and quantified using Image Lab™ software (Bio-Rad).

Statistical analysis
All results were repressed as means ± standard deviation (SD) cumulated from three independent experiments at least. Student’s t was used to compare the difference between groups. The one-way analysis of variance (ANOVA) method was conducted followed by Bonferroni’s test. Statistical significance was stated as p < .05.

Results
Quercetin mitigated hypoxia-caused cell viability reduction and apoptosis with rescuing activation of AMPK
To confirm a direct protective effect of quercetin on cell viability against hypoxia, we incubated H9c2 cells with quercetin before hypoxia stimulation. Cell viability analysis suggested that quercetin administration notably (p < .05 and p < .01) restored cellular viability, which was ameliorated by hypoxia (p < .01), in a dose-dependent manner (Figure 2(A)). However, of particular notice, 50 μM of quercetin treatment enhanced cell viability more slightly than 40 μM at least in part. Consequently, we treated H9c2 cells with 40 μM of quercetin in the downstream studies. Further, hypoxia stimulus elicited a conspicuous (p < .001) fortification in apoptotic progress, despite that, quercetin broadly (p < .01) blocked it (Figure 2(B)). Molecularly, hypoxia distinctly altered proteins profiles, especially, augmenting p53 level (p < .01), attenuating Bcl-2 generation (p < .01), and strengthening cleavage of caspase-3 (p < .001) (Figure 2(C)). Definitely, quercetin eliminated (p < .01) apoptosis (Figure 2(B)), meanwhile, restored apoptosis-relevant protein levels, including p53 (p < .05), Bcl-2 (p < .05), and cleaved caspase-3 (p < .01), shown in Figure 2(C). Additionally, phosphorylated AMPK down-regulation by hypoxia (p < .01) was prominently (p < .001) restored by quercetin (Figure 3). Cumulatively, quercetin has pleiotropic activities against hypoxia-caused H9c2 cells lesions, involving maintenance of cell viability and inhibition of apoptosis.

Quercetin flattened hypoxia-evoked overexpression of miR-199a in H9c2 cells
In particular, there is an evident (p < .001) amplification of miR-199a level in H9c2 cells which were stimulated in oxygen-deficient conditions (Figure 4). Moreover, we observed a noteworthy effect of quercetin on miR-199a, that is, quercetin (p < .01) ameliorated miR-199a accumulation exactly ascribed to hypoxia (p < .001) (Figure 4). Since quercetin mediated cell viability and apoptosis, with ameliorated miR-199a expression, one mechanism of its protection of quercetin against hypoxia-evoked lesions might as well be through modulation of miR-199a. To validate this hypothesis, we distinctly (p < .01) aggrandized miR-199a level by transducing exogenous miR-199a mimic into H9c2 cells (Figure 5(A)). In comparison with normal H9c2 cells, miR-199a-overexpressed cells exhibited (p < .05) weaker cell viability certainly in a precondition that these two cells were pre-incubated with quercetin before stimulated in a hypoxia circumstance (Figure 5(B)). As
for apoptotic progress, miR-199a overexpression ($p < .05$) apparently facilitated it in spite of quercetin treatment. This observation was consolidated by results from immunoblotting analysis about proteins implicated in apoptosis, including the ascension of p53 ($P < .05$), the decline of Bcl-2 ($p < .05$), along with cleavage of caspase-3 ($p < .05$) (Figure 5(D)). These features together with the presence of miR-199a in hypoxia-stimulating H9c2 cells might mark miR-199a as an incomparable potential target for the clinical development of agents against hypoxia-caused insults in cardiomyocytes.
Quercetin was implicated in modulation of sirt1 expression via abating miR-199a

Of importance, we found a target specificity for sirt1 3′-UTR at position sites 2816–2823 of its transcript (GI: 1519315080) by miR-199a (Figure 6(A)). Moreover, we found hypoxia environment remarkably eliminated sirt1 generation both at protein (Figure 6(B)) and (p < .01) mRNA levels (Figure 6(C)). By contrast, quercetin pre-treatment visibly (p < .001) restored its level. However, sirt1 level still (p < .01) remained reduced in miR-199a-replenished H9c2 cells even though they were pre-treated with quercetin before hypoxia stimulus (Figure 6(B,C)). One mechanism of sirt1 restoration by quercetin, proposed by us, might be inhibition of miR-199a-mediated post-transcriptional regulation. To validate whether sirt1 is a direct target gene of miR-199a, we performed the dual-luciferase reporter assay. For that, simultaneous transfection of miR-199a mimic or NC mimic and sirt1 (wt or mut) were conducted. The results showed that the luciferase activity was clearly inhibited by miR-199a mimic in sirt1-wt cells (p < .05), revealing that miR-199a directly bound to sirt1 3′-UTR (Figure 6(D)). These results suggested that quercetin was implicated in the modulation of sirt1 expression by abating miR-199a, which post-transcriptionally impeded translation progression of sirt1 by directly targeting its 3′-UTR.

AMPK phosphorylation was restored by quercetin-evoked up-regulation of sirt1

In order to further investigate the functional association between sirt1 and AMPK, sirt1-deficient H9c2 cells were stably established. As suggested in Figure 7(A,B), H9c2 cells significantly expressed sirt1 mRNA and protein (p < .001) both at a lower level compared with its NC. Stable silence of sirt1 resulted in a clear decline (p < .01) of AMPK as a phosphorylated form protein despite quercetin administration was carried out before hypoxia stimulation (Figure 7(C)). Collectively, our results demonstrated that quercetin played a role in the upregulation of sirt1 leading to the restoration of phosphorylated AMPK expression.
Discussion

Intensive studies were conducted to investigate the therapeutic significance of dietary agents. With notation to prove bioactivity of quercetin in cardiac hypoxia damage, H9c2 cells were subjected to quercetin administration before stimulated hypoxia circumstance. Moreover, it was confirmed that a possible mechanism associated with miR-199a-sirt1 was proposed in apoptotic progress.

In the present study, H9c2 cells were found to be more viable after quercetin pre-incubation as compared to the simple hypoxia-stimulation. In accordance, its cardio-protective roles were manifested by the significant decrease in apoptosis. In the mitochondrial pathway, through p53-dependent
apoptosis, stimulus evokes the release of apoptotic proteins from mitochondria by pro-apoptotic members of the Bcl-2 family, and ultimately leads to caspase activation [15]. As a consequence, p53, Bcl-2 and cleaved caspase-3 emerged as hallmarks of apoptosis in our study. AMPK is recognized as a serine-threonine kinase that primarily serves as a metabolic sensor, which is supposed to coordinate anabolic and catabolic activities [16]. Enhanced phosphorylation attenuates apoptosis [17] in response to hypoxia which might cause energy stress [18]. The cardioprotective effects of quercetin were suggested by restoring the phosphorylation of AMPK.

Intriguingly, H9c2 cells overexpressed miR-199a in response to hypoxia, while quercetin down-regulated miR-199a level at least in part. Obviously, the protective effects of quercetin were weakened in miR-199a-overexpressed cells. A growing body of evidence has shown that miR-199a is elevated in various models of heart failure [7,19]. Particularly, miR-199a participates in the mediation of metabolic progress in mitochondria. Cellular energy homeostasis is a fundamental status that entirely manages the cellular function, which is paramount to cellular survival [7]. Additionally, increased miR-199a has been verified to impair ubiquitin-proteasome system and then disrupt cardiomyocytes structure and impair endothelial cell function [20]. Our study exactly confirmed that it was through down-regulating miR-199a that quercetin exhibited a cardioprotective activity.

Furthermore, we considered that quercetin restored sirt1 synthesis by dampening miR-199a accumulation. Of special note, miR-199a directly targeted sirt1 3'-UTR, which is in concordance with a previous report [8] and indicates that miR-199a mediated sirt1 level dependent on post-transcriptional regulation. Sirt1 is a redox sensor and its interaction with hypoxia-inducible factor-1α is implicated in the crosstalk between oxygen and redox-responsive signaling transducers [21]. In response to oxidative stress, sirt1 normally positively regulates autophagy, increases mitochondria function and consequently reduces oxidative stress [22]. In concordance with our studies, it has been confirmed that by restoring sirt1 level antioxidants, such as polyphenols, possess activity to attenuate stimuli-caused lesions in cardiomyocytes [10,23–25]. Consequently, its cardiac protective function might be ascribed to its ability to restore sirt1 expression and then ameliorate hypoxia-induced mitochondrial oxidative damage.

Hypoxia stimulation notably resulted in a down-regulation of sirt1 while quercetin up-regulated sirt1 both at mRNA and protein level. Quercetin could directly scavenge free radicals [26], which might lessen oxidative stress. Sirt1 normally confers protection against ischemia/reperfusion damages in cardiomyocytes whereby different mechanisms [27–29]. In response to hypoxic stress, sirt1 facilitates autophagy via activating AMPK with attenuating hypoxia-evoked apoptosis [30]. Besides, in the cardiac aging-elicited compromise of myocardial function and morphology, AMPK phosphorylation and sirt1 activity are dampened by aldehyde dehydrogenase 2, an essential mitochondrial enzyme controlling cardiac function [31]. AMPK phosphorylation was found to be impeded in sirt1-silenced H9c2 cells, which were stimulated by quercetin before hypoxia stimulus. These results suggested that quercetin might exaggerate AMPK phosphorylation by restoring the sirt1 level.

In conclusion, our findings revealed that quercetin exhibited a profound cardio-protective ability via relieving apoptosis against hypoxic damages. Besides, we outlined a possible molecular mechanistic perception. Quercetin might re-establish a sirt1-AMPK-mediated defense mechanism by repressing miR-199a expression. These findings highlighted an avenue that quercetin could be administrated as a cardio-protective agent in the prevention or treatment of hypoxia-caused injury while more in vivo and clinical confirmations are required.

Disclosure statement
Authors declare that there is no conflict of interests.

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