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Quercetin attenuates vascular calcification by inhibiting oxidative stress and mitochondrial fission

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ABSTRACT

Vascular calcification is a strong independent predictor of increased cardiovascular morbidity and mortality and has a high prevalence among patients with chronic kidney disease. The present study investigated the effects of quercetin on vascular calcification caused by oxidative stress and abnormal mitochondrial dynamics both *in vitro* and *in vivo*. Calcifying vascular smooth muscle cells (VSMCs) treated with inorganic phosphate (Pi) exhibited mitochondrial dysfunction, as demonstrated by decreased mitochondrial potential and ATP production. Disruption of mitochondrial structural integrity was also observed in a rat model of adenine-induced aortic calcification. Increased production of reactive oxygen species, enhanced expression and phosphorylation of Drp1, and excessive mitochondrial fragmentation were also observed in Pi-treated VSMCs. These effects were accompanied by mitochondria-dependent apoptotic events, including release of cytochrome *c* from the mitochondria into the cytosol and subsequent activation of caspase-3. Quercetin was shown to block Pi-induced apoptosis and calcification of VSMCs by inhibiting oxidative stress and decreasing mitochondrial fission by inhibiting the expression and phosphorylation of Drp1. Quercetin also significantly ameliorated adenine-induced aortic calcification in rats. In summary, our findings suggest that quercetin attenuates calcification by reducing apoptosis of VSMCs by blocking oxidative stress and inhibiting mitochondrial fission.

1. Introduction

Vascular calcification, which increases with aging and is more prevalent in individuals with atherosclerosis, diabetes mellitus, and chronic kidney disease (CKD), is a strong independent predictor of increased cardiovascular morbidity and mortality [1–3].

Cell lineage tracing studies have demonstrated that vascular smooth muscle cells (VSMCs) play an important role in vascular calcification [4]. VSMCs usually remain in a quiescent differentiated state and play a central role in regulating vascular tone. When cultured *in vitro* in high phosphate medium or when exposed to high serum phosphate levels *in vivo* (*e.g.* in CKD patients with hyperphosphatemia), VSMCs can undergo calcification, leading to phenotypical changes and mineralization of their extracellular matrix [3,5–7]. It has also been demonstrated that apoptosis of VSMCs plays a crucial role in the development of VSMC calcification induced by inorganic phosphate (Pi). Both the apoptotic bodies derived from VSMCs and the matrix vesicles that originate from viable VSMCs can serve as nucleating structures for the formation of calcium phosphate crystals. Several studies have shown that vascular calcification can be attenuated by inhibiting apoptosis of VSMCs [5,6,8].

Mitochondria are organelles that play a key role in the life and death of eukaryotic cells. They perform diverse yet interconnected functions, producing ATP and many biosynthetic intermediates and also playing a central role in the intrinsic apoptotic pathway [9,10]. Mitochondria-mediated apoptosis involves the release of cytochrome *c* from the inner membrane space to the cytosol, which triggers sequential activation of the caspase-9 and -3 cascades [8]. Previous studies have demonstrated that these apoptotic events are closely related to mitochondrial dysfunction, including altered mitochondrial biogenesis, membrane potential depolarization caused by opening of the mitochondrial permeability transition pore, inhibition of electron transport leading to increased generation of oxidants and decreased intracellular ATP levels because of low respiratory activity caused by oxidant insult [8,11]. In the present study, we have confirmed that mitochondria are highly dynamic organelles that continuously divide and fuse to form new individual units and interconnected networks within the cell. A balance between mitochondrial fission and fusion is important for

Abbreviation: CKD, Chronic kidney disease; VSMCs, vascular smooth muscle cells; MGP, matrix Gla protein; Drp1, dynamin-like protein 1; ROS, reactive oxygen species; OGD, oxygen glucose deprivation; Pi, inorganic phosphate.

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mitochondrial function and form [9]. Excessive mitochondrial fission causes apoptosis in a number of pathological processes, including acute kidney injury [12], ischemia-reperfusion injury [13,14] and neuronal injury [15]. Mitochondrial fission and fusion are mediated by membrane-remodeling dynamin family proteins. In mammals, dynamin-like protein 1 (DLP1, also known as dynamin-related protein 1, Drp1) is the main protein regular of mitochondrial fission and also acts as an intrinsic factor in several mitochondria-dependent apoptosis pathways [16]. Drp1 silencing has been reported to reduce migration of VSMCs and to ameliorate intimal hyperplasia by altering mitochondrial energetics and levels of reactive oxygen species (ROS) [17]. Although Pi-induced vascular calcification has been linked to mitochondria-mediated apoptosis pathways [8], whether mitochondrial fission participates in the processes of vascular calcification is still unknown. Mitochondrial oxidative stress has also been identified as a primary cause of mitochondrial fragmentation *via* differential modulation of mitochondrial fission-fusion proteins [18]. Quercetin is an important antioxidant flavonoid that is present in a variety of fruits and vegetables. Besides scavenging free radicals, inhibiting xanthine oxidase and reducing lipid peroxidation, quercetin also exerts anti-inflammatory effects by suppressing the MAPK and NFκB signal transduction pathways [11,19]. Quercetin has been shown to modulate mitochondrial function in a number of diseases, including brain injury [20], cardiovascular diseases [21] and gastric inflammation [22].

The role of quercetin in Pi-induced vascular calcification caused by oxidative stress has not, however, been fully elucidated and the effect of quercetin on mitochondrial dynamics is still unclear. In the present study, our aim was to analyze the role of quercetin in preventing oxidative stress and improving mitochondrial structure and function during Pi-induced calcification of VSMCs. We also examined the effect of quercetin on mitochondrial dynamics, expression of the main proteins involved in mitochondrial fission and the relationship with Pi-induced apoptosis of VSMCs.

2. Materials and methods

2.1. Animals and ethical approval

Male Sprague Dawley rats (6–8 weeks old, 270–330 g) were purchased from WeiTong LiHua Experimental Animal Technology Company (Beijing, China). The rats were housed in a humidity-controlled room (25 ± 1 °C, 12 h light/dark cycle) and were provided with free access to water and food. All procedures were approved by the Institutional Animal Care and Use Committees at the First Affiliated Hospital of Harbin Medical University in Harbin, PR China and were conducted in compliance with National Institutes of Health guidelines for the care and use of laboratory animals.

2.2. Rat model of adenine-induced aortic calcification

An adenine-induced aortic calcification model was established in rats. Briefly, 6-week-old male rats in the experimental group were fed an adenine-rich diet and rats in the control group were fed a standard diet. Both the adenine-enriched diet and the standard diet were purchased from Keao XieLi Co., Ltd. (Beijing, China). The adenine-enriched diet contained adenine (0.75%), *Ca* (1.0%), Pi (1.2%), lactose (20%) and casein-based protein (19%). A solution of quercetin (Sigma–Aldrich Corp., St. Louis, MO, USA) in distilled water (100 mg/kg) was administered daily by gavage at 8:00 am, when the adenine-containing diet was supplied. After 4 weeks treatment, the rats were anaesthetized with 10% (v/v) chloral hydrate (350 mg/kg i.p.) and the whole aortas were collected by surgical dissection for determination of calcium content using von Kossa staining and hematoxylin and eosin (H&E) staining.

2.3. Cell culture experiments and Pi-induced calcification of VSMCs

Clonal embryonic rat aortic smooth muscle cells (A-10, Shanghai Yu Bo Biological Technology Co., Ltd., Shanghai, China) were maintained in complete growth medium (HyClone™ Dulbecco's modified Eagle's medium (GE Healthcare Life Sciences China, Beijing, China) containing 10% Gibco® fetal bovine serum (ThermoFisher Scientific, Waltham, MA, USA) and 100 ng/ml penicillin and streptomycin (Beyotime Biotechnology, Beijing, China)). To induce calcification, the A-10 cells were grown to sub-confluence and treated with calcification medium containing Pi (2.6 mM, pH 7.4) for 6 days. The mineralizing medium was further supplemented with quercetin (50–100 μM) or a selective Inhibitor of Drp-1, Midiv-1(5 μM, Selleck Chemicals, Houston, TX, USA), as indicated [23,24]. To investigate the effect of quercetin on VSMCs culture without Pi, we choose normal medium with quercetin (50–100 μM) as a negative control.

2.4. Measurement of calcium deposits

After induction of *in vitro* calcification, calcium deposition in the cells was visualized using alizarin red S staining, according to standard protocols [8]. For calcium quantification, 0.1 M HCl extracts of the cultured cells were analyzed colorimetrically using a QuantiChrom™ Calcium Assay Kit (BioAssay Systems, Hayward, CA, USA). After assaying, the remaining cells were washed three times with phosphate buffered saline and solubilized in RIPA Lysis Buffer (Solarbio Life Sciences, Beijing, China). The protein concentration was estimated using an Enhanced BCA Protein Assay Kit (Beyotime Biotechnology). The calcium content was normalized to protein concentration.

2.5. Measurement of mitochondrial membrane potential and intracellular ATP concentration

VSMCs (clonal embryonic rat aortic smooth muscle cells) were seeded in tissue culture dishes (60 mm surface diameter) at a density of 1×10^6 cells per dish, grown to confluence and then further incubated in the absence or presence of Pi or quercetin for 3 days. To assess mitochondrial membrane potential, cells were loaded with JC-1 dye (2 μM, MitoProbe™ JC-1 Assay Kit for Flow Cytometry, Thermo Fisher Scientific) for 30 min, according to manufacturer's instructions. At least 10,000 events of the stained cells were analyzed using a FACSAria flow cytometer (Becton, Dickinson and Company, Lake Franklin, NJ, USA), with an excitation wavelength of 488 nm and an emission wavelength of 530 nm. The fluorescence index was represented as fold-difference relative to control.

ATP levels were determined using a luciferase-based Enzylight™ ATP Assay Kit (BioAssay Systems), according to the manufacturer's instructions. ATP concentrations were normalized to the protein concentration of the resulting supernatant, determined using an Enhanced BCA Protein Assay Kit (Beyotime Biotechnology).

2.6. Assessment of apoptosis using flow cytometry

Following the treatments described above, the cells were washed with ice-cold PBS, harvested and counted. The cells (1×10 [5]) were suspended in binding buffer (500 μl) containing HEPES/NaOH (0.1 M, pH 7.4), (NaCl 1.4 M) and $CaCl_2$ (25 mM) and incubated with Annexin V-FITC (10 μl) and PI (5 μl, FITC Annexin V Apoptosis Detection Kit I, Becton, Dickinson and Company) for 20 min. The rate of apoptosis (%) was measured using a FACSAria flow cytometer.

2.7. Determination of oxidant generation

Cells (~0.5×10^6) were plated in tissue culture dishes (60 mm surface diameter) and incubated in the absence or presence of Pi and different concentrations of quercetin for 3 days. The cells were further