Combination treatment with quercetin and resveratrol attenuates high fat diet-induced obesity and associated inflammation in rats via the AMPKα1/SIRT1 signaling pathway

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Abstract. Diet-induced obesity is associated with systemic inflammation, which is considered to originate predominantly from the adipose tissue. Quercetin and resveratrol are two dietary polyphenols that exhibit anti-inflammatory properties and anti-insulin resistance when administered in isolation or combination (CQR). It remains unknown whether CQR reduces high fat diet (HFD)-induced obesity and inflammation in rats. In the current study, 46 male Wistar rats were divided into two groups, one of which was fed a normal diet (ND, 5.4% fat, w/w) and one of which was fed a HFD (45% fat, w/w) for 3 weeks. Following removal of the 12 most obesity-resistant rats from the HFD group, the remaining rats were divided into two sub-groups: A HFD group and a HFD+CQR group (administered 120 mg/kg/day resveratrol and 240 mg/kg/day quercetin). The results revealed that the HFD+CQR group had significantly lower body weights at 11 weeks compared with the HFD group and had significantly reduced visceral adipose tissue weights and adipocyte sizes. Serum lipid profiles were also significantly ameliorated in the HFD+CQR group. CQR attenuated the expression of systemic proinflammatory adipokines, including leptin, tumor necrosis factor-α, monocyte chemoattractant protein-1 and interleukin-6. It also reduced the recruitment of mast cells to the epididyotic adipose tissue (EAT). Furthermore, CQR reversed the HFD-induced suppression of 5'-adenosine monophosphate-activated protein kinase α1 (AMPKα1) phosphorylation and sirtuin 1 (SIRT1) expression in EAT. In conclusion, CQR may suppress obesity and associated inflammation via the AMPKα1/SIRT1 signaling pathway in rats fed a HFD.

Introduction

Obesity-induced systemic inflammation originates in adipose tissue prior to hepatic tissue (1,2). The human body contains various fat deposits, which can be divided into white and brown fat. White adipose tissue (WAT) is a multifunctional organ that stores nutrients in the form of fat droplets. In addition, WAT secretes cytokines that affect the body's metabolic state, thus it is sometimes regarded as the largest endocrine organ in the body (3). Excessive energy intake induces adipocyte hypertrophy and hyperplasia, which may lead to the development of high fat diet-induced obesity. Hypertrophic adipocytes release chemokines and proinflammatory cytokines to activate and attract inflammatory cells into WAT. This contributes to systemic insulin resistance and ultimately, a state of chronic low-grade adipose tissue inflammation (4). Polyphenol intake is positively associated with a decrease in the incidence of metabolic and obesity-associated disorders. Quercetin is a polyphenolic flavonoid compound present in a variety of fruits and vegetables, including onions, broccoli, tomatoes, apples and berries. It has a wide range of biological activities and health-promoting effects, including anti-carcinogenic (5), antiviral (6), antioxidant (7), anti-diabetic (8), anti-inflammatory (9), anti-aging (10) and angioprotective properties (11). Furthermore, it has recently been suggested that quercetin exerts anti-obesity activity via the mitogen-activated protein kinase (MAPK) and 5'-adenine

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monophosphate-activated protein kinase α1 (AMPKα1) signaling pathways (12). Resveratrol, a phytoalexin found in the skin and seeds of grapes and in red wine, may also protect against diet-induced obesity and metabolic diseases including hepatic steatosis and insulin resistance (13). In the present study, the effect of combination treatment with quercetin and resveratrol (CQR) was investigated in rats fed a HFD. The impact on CQR on HFD-induced fat accumulation, insulin resistance, proinflammatory cytokine levels, mast infiltration and AMPKα1/sirtuin 1 (SIRT1) signaling in adipose tissues was assessed.

Materials and methods

Animals. The present study was approved by the Institutional Animal Care and Use Committee of Shanghai University of Traditional Chinese Medicine (Shanghai, China), and all procedures were performed in accordance with the National Institute of Health's guidelines (14). A total of 46 male 8-week-old Wistar rats with a mean weight of 200±10 g were provided by the Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). Rats were raised in an environment of 22±0.5°C and 40-70% relative humidity under a 12 h light/dark cycle, with food and water freely available. Following 1-week habituation, the rats were randomly divided into 2 groups. The normal diet (ND) group, (n=10) were fed a regular chow diet containing 5.4% fat and the HFD group, (n=36) were fed a HFD containing 45% fat. After 3 weeks, rats in the HFD group were ranked according to weight gain, and rats in the lower third (n=12) were excluded from the study as they were deemed to be obesity resistant. The remaining 24 rats in the HFD group were randomly divided into 2 sub-groups: i) A HFD group (n=12); and ii) a HFD+CQR (n=12) group. CQR treatment consisted of 120 mg/kg/day resveratrol (purity, ≥98%; Hangzhou Great Forest Biomedical Co., Ltd., Hangzhou, China) and 240 mg/kg/day quercetin (purity ≥98; Nanjing Zelang Medical Technology Co., Ltd., Nanjing, China) The body weight and food intake of the rats was recorded each week. After 11 weeks, rats were anesthetized with isoflurane and sacrificed following a 12 h fast. Blood was extracted from the abdominal aorta and centrifuged at 5,000 x g for 15 min at 4°C. The serum was separated and stored at -80°C prior to analysis. Following blood collection, subcutaneous adipose tissues (SATs), epididymis adipose tissues (EATs) and perinephric adipose tissues were promptly removed, rinsed, weighed on ice and then snap frozen in liquid nitrogen and stored at -80°C.

Biochemical parameters. Total cholesterol (C), triglycerides (TG), high-density lipoprotein-C (HDL-C) and low-density lipoprotein-C (LDL-C) in the serum were measured and quantified using a Hitachi 7600 automatic biochemical analyzer (Hitachi, Ltd., Tokyo, Japan) with the corresponding kits as follows: Quick Auto Neo T-CHOLII assay reagent and Quick Auto Neo TGII assay reagent, manufactured by Shino-Test Corporation (Tokyo, Japan); L-type HDL-C reagent 1 (cat no. 998-09011), reagent 2 (cat no. 994-09111); and L-type LDL-C reagent 1 (cat no. 997-39893) and reagent 2, (cat no. 993-39993), manufactured by Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Cytokine quantification. Levels of leptin, adiponectin, insulin, interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) and monocyte chemotactic protein-1 (MCP-1) in the serum were quantified using the corresponding commercial rat ELISA kits as follows: Rat leptin, LEP ELISA kit (cat no. CSB-E07433r); rat adiponectin, ADP ELISA kit, (cat no. CSB-E07271r); rat insulin, INS ELISA kit (CSB-E05070r); rat IL-6, IL-6 ELISA kit (cat no. CSB-E04640r); rat TNF-α ELISA kit (cat no. CSB-E11987r); and rat MCP-1/monocyte chemo-tactic and activating factor, MCP-1/MCAF ELISA kit (cat no. CSB-E07429r; Cusabio Biotech Co., Ltd., Wuhan, China) according to the manufacturer's protocols.

Histopathology. Adipose tissues were collected at 11 weeks and fixed in 4% formalin at room temperature for 24 h, embedded in paraffin and sliced serially into sections 5-μm thick. To determine adipocyte size, hematoxylin and eosin staining was conducted on EATs at room temperature for 30 min. Five visual fields were randomly selected from each section with an Olympus BX51 light microscope (Olympus Corporation, Tokyo, Japan) and examined using Image-Pro Plus version 6.0 (Media Cybernetics, Inc., Rockville, MD, USA) to determine the average adipocyte diameter. Toluidine blue staining was also performed on EATs for 1 h by briefly submerging tissue sections in 0.1% aqueous toluidine blue (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at room temperature, the histological images were used to quantify the number of mast cells present, as previously described (15). Mast cell numbers were counted using a light microscope and were presented as cell numbers/mm^2.

Protein extraction and western blot analysis. Plasma membrane proteins were extracted from adipose tissues using a radioimmunoprecipitation assay lysis buffer (cat no. 89900, Pierce; Thermo Fisher Scientific, Inc., Waltham, MA, USA), total protease inhibitor and phosphatase inhibitor, as described previously (16). Protein concentration was measured using a commercial bicinchoninic acid assay kit and a microplate reader at 570 nm. Subsequently protein (40 μg/lane) was separated by 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membranes were blocked with 2% bovine serum albumin (cat no. K720; Ameresco, Inc., Framingham, MA, USA) at room temperature and TBS with Tween-20 for 1 h, then incubated overnight at 4°C with AMPKα1 antibody (cat no. 2795), phosphorylated (p)-AMPKα1 (Thr172; cat no. 2535), or SIRT1 rabbit monoclonal antibodies (cat no 3931). Membranes were also incubated with monoclonal mouse anti-human β-actin antibody (cat no. 4967; all 1:1,000 dilution; all from Cell Signalling Technology, Inc., Danvers, MA, USA) as the loading control. Following extensive washing in Tween-PBS, membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G antibody (1:4,000; cat no. sc-2030; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for 1 h. Bands were visualized using LightShift™ Chemiluminescent electrophoretic mobility shift assay kit (cat no. 20148; Thermo Fisher Scientific, Inc.) and the ImageQuant™ LAS 4000 Mini, quantified using ImageQuant TL 7.0 software (both from GE Healthcare, Chicago, IL, USA) and expressed as the ratio of pAMPKα1 to AMPKα1 or SIRT1 to β-actin.
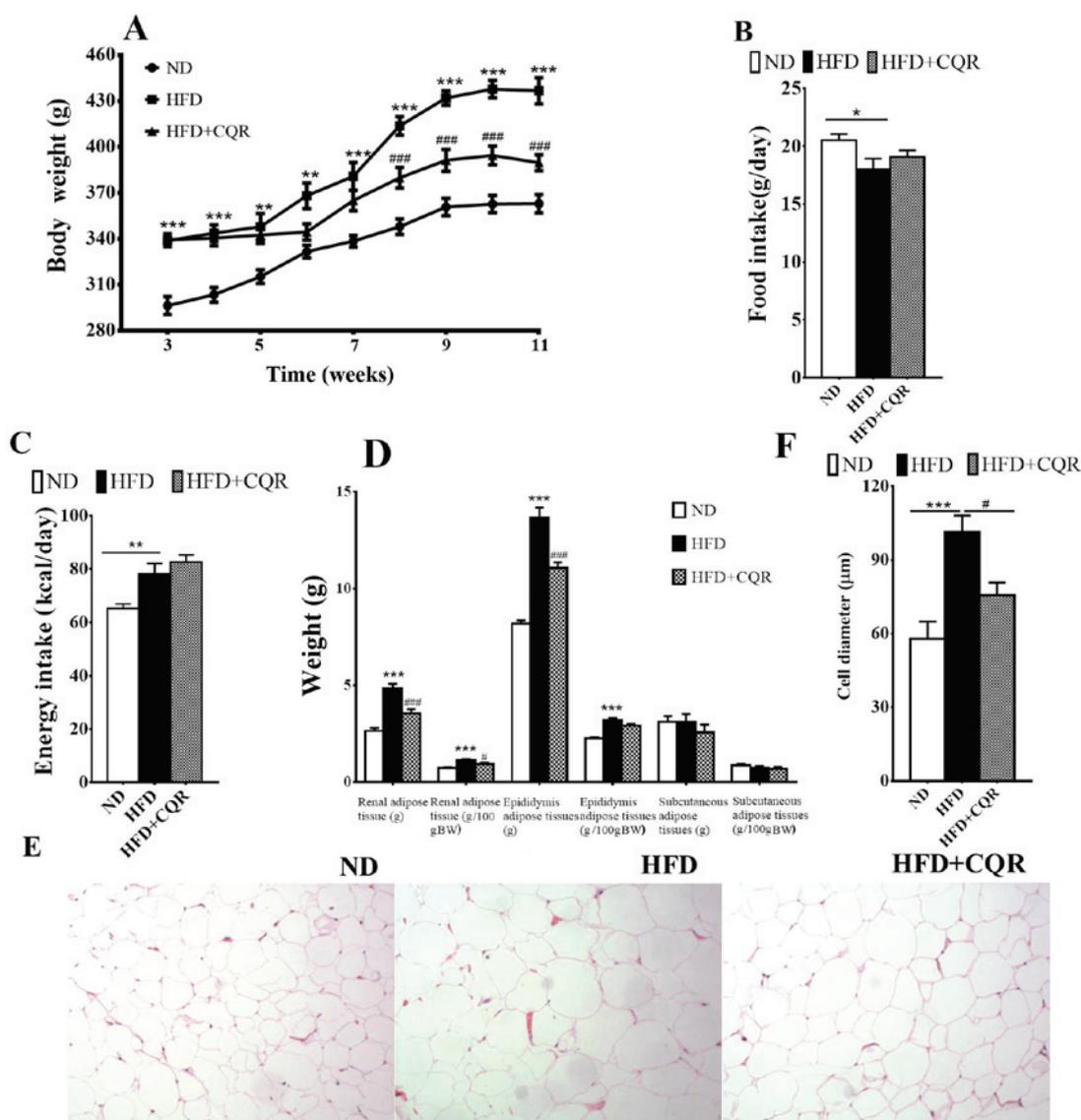
Figure 1. Treatment with CQR reduces the body and visceral fat weights of rats fed a HFD. Rats were fed a ND, a HFD or a HFD+CQR for a period of 11 weeks. Subsequently (A) body weight, (B) food intake, (C) energy intake and (D) white adipose tissue weights were measured. (E) EAT morphology was observed using a light microscope at a magnification of x200 and the (F) adipocyte diameters in EAT were measured. Data are expressed as the mean ± standard error of mean. *P<0.05, **P<0.01 and ***P<0.001 vs. ND. #P<0.05 and ###P<0.001 vs. HFD. CQR, combination of quercetin and resveratrol; HFD, high fat diet; ND, normal diet; EAT, epididymal adipose tissues.

Statistical analysis. All data are presented as the mean ± standard error of the mean. For multiple comparisons, differences were analyzed using one-way analysis of variance followed by Tukey's multiple comparison test. P<0.05 was considered to indicate a statistically significant difference. All statistics were analyzed using Graphpad Prism version 6.0 (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Treatment with CQR results in a lower body and visceral adipose tissue weight in an HFD-rat model. After 3 weeks, at the point of CQR intervention, the body weight of rats in the HFD group was significantly higher than those in the ND group (Fig. 1A). The body weight increase was alleviated by CQR between weeks 8 and 11 and following 11 weeks intervention; HFD+CQR rats had a significantly lower body weight

than HFD rats (Fig. 1A). Food intake was significantly lower in HFD rats compared with ND rats (Fig. 1B), however energy intake was significantly higher (Fig. 1C). CQR did not exert a marked effect on food and energy intake. At the end of week 11, HFD+CQR rats had a notably lower visceral (epididymal and perirenal) adipose tissue weight (Fig. 1D) and a significantly smaller adipose cell diameter compared with the HFD group (Fig. 1E and F). Subcutaneous adipose tissue weight did not differ significantly across all groups (Fig. 1D). These results suggest that CQR treatment may inhibit HFD-induced obesity.

CQR treatment affects lipid levels in the serum. The HFD group exhibited significantly higher levels of total C, TG and LDL-C in the serum compared with the ND group (Table I). CQR significantly attenuated these lipid levels compared with the HFD. However, CQR did not reverse the decrease in serum HDL-C induced by HFD (Table I).

Table I. CQR normalizes the concentrations of serum constituents in HFD fed rats.

Parameter	ND	HFD	HFD+CQR
Serum total C (μmol/l)	1536±101.5[a]	1987±100.6	1587±70.36[a]
Serum TG (μmol/l)	685.8±89.93[c]	1366±129.3	746.4±80.98[c]
Serum HDL-C (μmol/l)	1010±24.72[b]	778.3±30.79	901.7±23.09
Serum LDL-C (μmol/l)	275.0±13.76[b]	462.5±29.39	305.0±31.66[b]
Serum insulin (IU/ml)	66.66±4.422[a]	84.65±4.917	68.16±3.454[a]
Serum leptin (pg/ml)	542.5±27.07[c]	904.2±47.88	667.7±36.90[c]
Serum adiponectin (pg/ml)	68.78±4.889[a]	38.74±3.740	60.70±3.934
Serum TNF-α (pg/ml)	41.50±6.000[b]	71.28±3.545	50.34±5.403[a]
Serum IL-6 (pg/ml)	1.141±0.0871[b]	2.063±0.2744	1.131±0.0842[b]
Serum MCP-1 (pg/ml)	47.56±5.594[a]	72.47±3.848	49.01±4.671[a]

Statistical differences between groups were identified using a one-way ANOVA test followed by Tukey's multiple comparison test, n=12 per group. All data in the table are presented as the mean ± standard error of mean. [a]$P<0.05$, [b]$P<0.01$ and [c]$P<0.001$, vs. the HFD model group. C, cholesterol; TG, triglycerides; HDL, high-density lipoprotein; LDL, low-density lipoprotein; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; MCP-1, monocyte chemotactic protein-1; ND, normal diet; HFD, high fat diet; CQR, combination of quercetin and resveratrol.

CQR treatment reduces insulin and leptin levels in serum. HFD significantly elevated serum insulin and leptin levels but lowered the serum adipinectin levels compared with the ND (Table I). CQR significantly decreased serum insulin and leptin levels but exhibited no significant effect on serum adipinectin compared with the HFD group.

CQR suppresses the clustering of mast cells in adipose tissue. During the formation of HFD induced-obesity and exacerbation of adipose tissue inflammation or insulin resistance, a large amount of mast cells infiltrate the adipose tissue (17). It was reported that HFD induced mast cell clustering in WAT and promoted obesity and insulin resistance (18), while quercetin suppresses the release of cytokines from mast cells *in vitro* and obesity mice (16,19). The number of mast cells in EAT was calculated to evaluate the effects of CQR on mast cell in adipose tissue of HFD-induced rats (Fig. 2). The results demonstrated that HFD significantly promoted the transition of mast cells into EATs, while CQR significantly reversed this effect (both P<0.05).

CQR suppresses proinflammatory cytokines. A variety of proinflammatory cytokines are secreted by hypertrophic adipocytes and cause inflammatory cell infiltration during the development and progression of obesity (20). Several important proinflammatory cytokines involved in insulin resistance were detected in this study; results from ELISA determined that levels of the proinflammatory cytokines TNF-α, IL-6 and MCP-1, which increased in rats on a HFD, were significantly suppressed by CQR (Table I). These results suggest that CQR may relieve systematic inflammation induced by obesity.

CQR upregulates the AMPKα1/SIRT1 signalling pathway. AMPKα1 and SIRT1 are two key nutrient sensors linking nutrient metabolism and inflammation (21-22). AMPKα1 negatively regulates lipid-induced inflammation, which acts through SIRT1 to protect against obesity, inflammation and insulin resistance (23). It has been demonstrated that quercetin
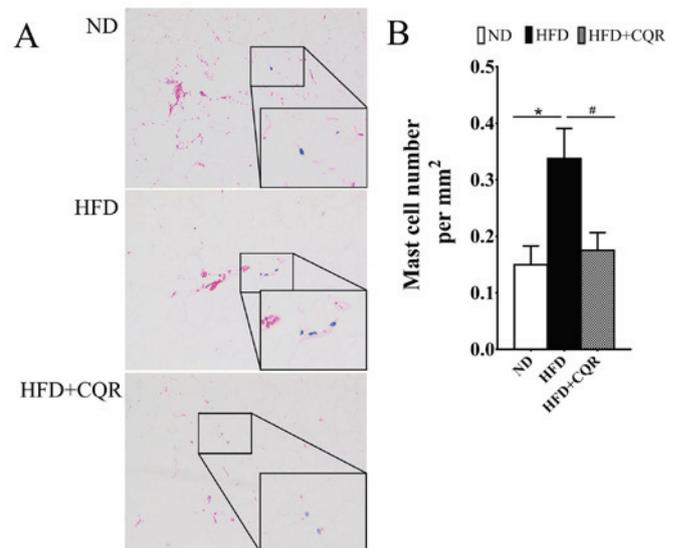

Figure 2. Treatment with CQR reduces the clustering of mast cells in the epididymal adipose tissue (EAT). At 11 weeks, tissue samples were collected and (A) epididymal white adipose tissue was observed using a light microscope (small box: magnification, x200, big box: magnification, x100) following toluidine blue staining of the mast cells indicated by the blue stain. Pink plaques are erythrocytes and leukocytes in the blood vessels of EAT. Erythrocytes are cells without a nucleus, while leukocytes are cells with a nucleus. (B) Quantification of mast cells in the indicated groups was performed by observing the slides, n=8 per group. [*]$P<0.05$ and [#]$P<0.05$. All data are presented as the mean ± standard error of mean. CQR, combination of quercetin and resveratrol; HFD, high fat diet; ND, normal diet.

alleviates obesity-associated adipose tissue macrophage infiltration and inflammation in mice via the AMPKα1/SIRT1 signaling pathway (16). Resveratrol also induces beneficial effects on obesity and metabolic disturbances by activating the AMPKα1/SIRT1 signaling pathway (24). Consistent with previous studies, AMPKα1 phosphorylation (Fig. 3A) and SIRT1 expression (Fig. 3B) in the EAT of rats fed a HFD were significantly suppressed. Treatment with CQR significantly
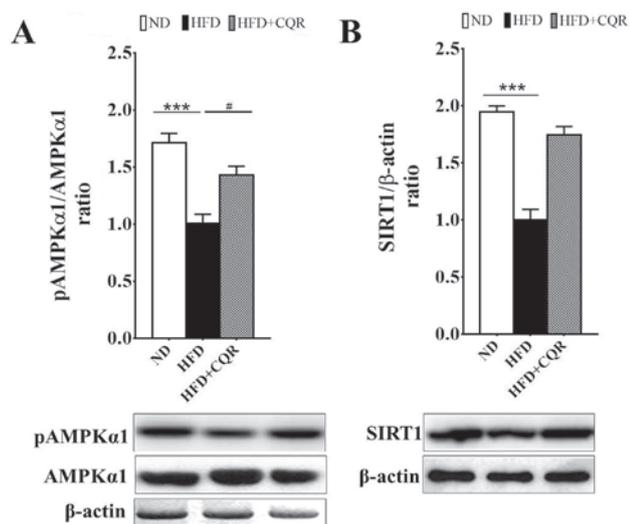
Figure 3. Treatment with CQR increases AMPKα1 phosphorylation and SIRT1 expression in the EAT of rats fed a HFD. After 11 weeks, tissue samples were obtained from each group and the (A) protein and phosphorylation levels of AMPKα1 and (B) the protein expression of SIRT1 in EATs, were measured. Quantification of AMPKα1 activity and SIRT1 expression was presented as the ratio of pAMPKα1 to total AMPKα1 and SIRT1 to β-actin, respectively. Statistical differences between groups were identified using a one-way ANOVA test followed by Tukey's multiple comparison test (n=8 per group). All data are presented as the mean ± standard error of mean. ***$P<0.001$ and #$P<0.05$. AMPKα1, 5'-adenosine monophosphate-activated protein kinase α1; SIRT1, sirtuin 1; EAT, epididymal adipose tissue; HFD, high fat diet; ND, normal diet; CQR, combination of quercetin and resveratrol; p, phosphorylated.

reversed the suppression of AMPKα1 phosphorylation in a rat model (Fig. 3A).

Discussion

Quercetin and resveratrol are two types of dietary polyphenols, which may have beneficial effects on metabolic syndrome (25-33). It has also been reported that quercetin and resveratrol have a therapeutic effect on triacylglycerol metabolism in WAT (25). The results of the present study suggest that the combination of quercetin and resveratrol ameliorate insulin resistance and adipose tissue inflammation in obese rats. Furthermore, CQR treatment was able to reverse the changes in AMPKα1 phosphorylation and SIRT1 expression that occur in adipose tissues. To the best of our knowledge, the current study is the first to demonstrate that CQR has synergic effects on body fat accumulation and adipose inflammation by activating the AMPKα1/SIRT1 signaling pathway.

Previous studies have demonstrated that resveratrol and quercetin may reduce body fat accumulation in animal models (26-31). In our previous study, CQR exhibited synergistic effects on a HFD-induced metabolic phenotype in mice (32). Since polyphenols are efficient, particularly at higher doses (33), doses of 120 mg/kg/day resveratrol and 240 mg/kg/day quercetin were used in the current study. These are similar to the doses used in other comparable studies performed in rodents (31,34-36). The recommended dosage of quercetin and resveratrol in obesity or insulin-resistance studies remains controversial and it was reported that treatment with lower doses of resveratrol may activate SIRT1,

whereas higher doses activate AMPK in a SIRT1-independent manner (37). The doses of quercetin and resveratrol used in some studies are 30 mg/kg/day and 15 mg/kg/day, respectively (26,38). In addition, quercetin is considered to be safe as it does not induce carcinogenicity and genotoxicity following oral administration at high doses (up to 2,000 mg/kg) (39). Lagouge et al (31) demonstrated that dietary treatment with 200 or 400 mg/kg/day resveratrol, delivered in either chow or a HFD, significantly increased the aerobic capacity and resistance to HFD induced-obesity in mice.

The anti-obesity effects of quercetin and resveratrol on HFD-induced body weight gain remain controversial and negative results have been reported by several research groups (40-42). Similar to previous studies (24,26,29,32,43), the results of the present study demonstrated that the body weight gain between weeks 9 and 11 in rats fed a HFD and treated with CQR was significantly attenuated compared with rats fed a HFD alone. Treatment with CQR also reduced the weights of renal adipose tissues and EATs, however it did not affect SAT weight or food and energy intake. CQR therefore appears to have a mild weight-reducing and visceral fat-reducing effect. Visceral adipose tissue is a proinflammatory endocrine tissue and may be responsible for the increased cardiometabolic risk that occurs as body mass index rises (44). Regardless of adiposity status, visceral adiposity is associated with an adverse cardiometabolic profile, including inflammation, insulin resistance and myocardial dysfunction, all of which are hallmarks of an 'obese' phenotype (44). In addition the current study demonstrated that, HFD-induced dyslipidemia caused an increase in total C, TGs and LDL-C and a decrease in HDL-C in the blood. Chaudhari et al (45) also reported that a HFD induced significant increases in total C, TG and LDL-C in the rat serum, whereas it reduced HDL-C in rat serum (41). In the present study, CQR treatment increased serum HDL-C and decreased serum TC, TG and LDL-C, demonstrating that CQR is able to reduce the effects of a HFD in a rat model of obesity, which is consistent with previous reports (12,46-48).

Chronic low-grade adipose tissue inflammation serves an important role in the development of HFD-induced obesity and insulin resistance (4,49,50). The proinflammatory or anti-inflammatory molecules abnormally secreted from obese adipose tissue are called adipokines and provide evidence that there is a direct association between obesity and systemic inflammation (51). The adipose tissue of obese individuals exhibits increased expression of proinflammatory adipokines, including TNF-α, MCP-1 and IL-6 but a reduced expression of adiponectin (52). Systemic leptin is increased in animals fed a HFD or with inflammation and/or infection states and directly affects cytokine production (53); thus it was hypothesized that CQR may ameliorate the inflammation in adipose tissue induced by a HFD. The results of the current study demonstrated that CQR attenuates adipocyte growth in EAT and mast cell clustering into adipose tissues. In the serum, CQR treatment reduced leptin, as well as TNF-α, MCP-1 and IL-6 levels.

To reveal the molecular mechanisms by which CQR attenuates obesity associated adipose tissue inflammation, two important nutrient sensors and inflammatory regulators in EAT were assessed in the current study; AMPKα1 and SIRT1 (54,55). CQR significantly increased

HFD-suppressed AMPKα1 phosphorylation and markedly increased SIRT1 expression in EATs, suggesting that CQR influences the AMPKα1/SIRT1 signaling pathway in adipose tissues. The AMPKα1/SIRT1 signaling pathway may be a novel cellular target due to its anti-inflammatory effects in adipocytes (24,56). AMPKα1 activates SIRT1 and inhibits inflammation in macrophages (57); furthermore, AMPKα1 may inhibit the activation of the nuclear factor-κB system, a key regulator of innate immunity and inflammation (55). Activation of AMPKα1 may suppress the synthesis of pro-inflammatory cytokines, such as IL-6 and IL-8, in adipocytes (58). Aminoimidazole-4-carboxami-deriboside, a pharmacological activator of AMPKα1, may inhibit inflammatory responses via AMPKα1-independent pathways, and reverse lipopolysaccharide and HFD-induced inflammation (55-59). Reduction of EAT SIRT1 expression may induce ectopic inflammatory gene expression and the overexpression of SIRT1 inhibits HFD-induced increases in inflammation in adipose tissue (60). Dong *et al* (16) reported that dietary quercetin suppressed adipose tissue macrophage infiltration and inflammation via the AMPKα1/SIRT1 pathway in mice fed a HFD. Furthermore, Bitterman and Chung (61) reported that the AMPKα1/SIRT1 signaling pathway is the primary target for the metabolic effects of resveratrol.

In conclusion, the results of the present study suggest that CQR ameliorates not only excessive body weight gain and dyslipidemia, but also adipose tissue inflammation in rats with HFD-induced obesity. The anti-obese effect of CQR is associated with a reduction in body weight gain, adipocyte diameter, adipose tissue weight and an improvement of dyslipidemia in serum. Its anti-obese effect is closely associated with its anti-inflammation effects by which it reduces adipokine secretion and activates the AMPKα1/SIRT1 signaling pathway. These results indicate that CQR has the potential to reduce HFD-induced obesity and inflammation.

Acknowledgements

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