Quercetin attenuates adipose hypertrophy, in part through activation of adipogenesis in rats fed a high-fat diet

Diahann J. Perdicaro, Cecilia Rodriguez Lanzi, Julián Gambarte Tudela, Roberto M. Miatello, Patricia I. Oteiza, Marcela A. Vazquez Prieto

PII: S0955-2863(19)30547-9

DOI: https://doi.org/10.1016/j.jnutbio.2020.108352

Reference: JNB 108352

To appear in: *The Journal of Nutritional Biochemistry*

Received date: 28 May 2019

Revised date: 22 December 2019

Accepted date: 17 January 2020

Please cite this article as: D.J. Perdicaro, C.R. Lanzi, J.G. Tudela, et al., Quercetin attenuates adipose hypertrophy, in part through activation of adipogenesis in rats fed a high-fat diet, *The Journal of Nutritional Biochemistry*(2020), https://doi.org/10.1016/j.jnutbio.2020.108352

Quercetin attenuates adipose hypertrophy, in part through activation of adipogenesis in rats fed a high-fat diet

Diahann J. Perdicaro[a1], Cecilia Rodriguez Lanzi[a1], Julián Gambarte Tudela[b], Roberto M. Miatello[a], Patricia I. Oteiza[c], Marcela A. Vazquez Prieto[a]*.

[1]These authors contributed equally to this work.

[a]Laboratorio de Nutrición y Fisiopatología de la Obesidad y [b]Laboratorio de Bioquímica e Inmunología, Instituto de Medicina y Biología Experimental de Cuyo (IMBECU)-CONICET y Facultad de Ciencias Médicas, Universidad Nacional de Cuyo, Mendoza, Argentina.

[c]Departments of Nutrition and Environmental Toxicology, University of California, Davis, USA.

*Corresponding author: Marcela A. Vazquez Prieto, School of Medicine, National University of Cuyo and IMBECU-CONICET, Av. Libertador 80 M5502JMA Mendoza, Argentina, Phone: ++54 261 4135000 ext. 2686 / Fax: ++54 261 4494047. mvazquez@fcm.uncu.edu.ar

Abstract

An impaired capacity of adipose tissue expansion leads to adipocyte hypertrophy, inflammation and insulin resistance (IR) under positive energy balance. We previously showed that a grape pomace extract, rich in flavonoids including quercetin (Q), attenuates adipose hypertrophy. This study investigated whether dietary Q supplementation promotes adipogenesis in the epididymal white adipose tissue (eWAT) of rats consuming a high-fat diet, characterizing key adipogenic regulators in 3T3-L1 pre-adipocytes. Consumption of a high-fat diet for 6 weeks caused IR, increased plasma TNFα concentrations, eWAT weight, adipocyte size and the eWAT/brown adipose tissue (BAT) ratio. These changes were accompanied by decreased levels of proteins involved in angiogenesis, VEGF-A and its receptor 2 (VEGF-R2), and of two central adipogenic regulators, i.e. PPARγ and C/EBPα, and proteins involved in mature adipocyte formation, i.e. fatty acid synthase (FAS) and adiponectin. Q significantly reduced adipocyte size and enhanced angiogenesis and adipogenesis without changes in eWAT weight and attenuated systemic IR and inflammation. In addition, high-fat diet consumption increased eWAT hypoxia inducible factor-1 alpha (HIF-1α) levels and those of proteins involved in adipose inflammation (TLR-4, CD68, MCP-1, JNK) and activation of endoplasmic reticulum (ER) stress, i.e. ATF-6 and XBP-1. Q mitigated all these events. Q and quercetin 3-glucoronide prevented TNFα-mediated downregulation of adipogenesis during 3T3-L1 pre-adipocytes early differentiation. Together, Q capacity to promote a healthy adipose expansion enhancing angiogenesis and adipogenesis may contribute to reduced adipose hypertrophy, inflammation and IR. Consumption of diets rich in Q could be

useful to counteract the adverse effects of high-fat diet-induced adipose dysfunction.

Graphical Abstract

Abbreviations: BAT, brown adipose tissue**; C/EBP**, CCAAT/enhancer-binding protein; **eWAT**, epididymal white adipose tissue; **ER**, endoplasmic reticulum; **FAS,** fatty acid synthase; **G**, quercetin 3-glucuronide; **HFD**, high-fat diet group; **HFD+Q**, high-fat diet group plus quercetin; **HIF-1α**, hypoxia inducible factor-1 alpha; **IR**, insulin resistance; **MCP-1**, monocyte chemotactic protein-1; **PPARγ**, peroxisome proliferator-activated receptor gamma; **Q**, quercetin; **TLR-4**, toll like type receptor 4; **TNFα**, tumor necrosis factor alpha; **VEGF**, vascular endothelial growth factor; **VEGF-R2**, vascular endothelial growth factor-receptor 2.

Keywords quercetin**;** adipogenesis; angiogenesis; inflammation; adipose tissue

1. Introduction

Obesity is one of the main public health concerns worldwide [1, 2]. Increased adiposity, mainly visceral adipose tissue, is strongly associated with insulin resistance (IR), type 2 diabetes and cardiovascular disease, among others. In the presence of excessive caloric intake, the fat mass increases as a consequence of an increase of adipocyte number (hyperplasia) and/or size (hypertrophy). It is well established that adipocyte hypertrophy is a major characteristic of adipose tissue dysfunction [3]. Adipocyte hypertrophy activates

redox signaling cascades leading to the up-regulation of pro-inflammatory adipocytokine expression (e.g., tumor necrosis factor alpha (TNFα), monocyte chemotactic protein-1 (MCP-1), Interleukin-6 and organelle stress (e.g., endoplasmic reticulum (ER) stress) leading to adipose dysfunction [4]. This pro-inflammatory scenario contributes to the recruitment and infiltration of macrophages. Macrophages express the Toll like receptors-4 (TLR-4), an innate immune receptor that binds to endogenous and exogenous ligands such as lipopolysaccharides. TLR-4-ligand binding leads to the activation of the nuclear transcription factor kappa B (NF-κB) that promotes the transcription of various genes involved in the inflammatory response contributing to perpetuate the inflammatory scenario in adipose tissue and adiposity-associated systemic IR [5].

Adipogenesis is the process by which precursor stem cells differentiate into mature adipocytes. It involves the activation of signaling cascades which coordinate the expression of genes responsible for adipocyte differentiation [6]. Both peroxisome proliferator-activated receptor gamma (PPARγ) and CCAAT/enhancer-binding proteins (C/EBP) are central adipogenic regulators that positively control each other and cooperate in adipose tissue differentiation. In addition, fat expansion requires an adequate vascularization to enable tissue delivery of oxygen and nutrients. The vascular endothelial growth factor-A (VEGF-A) acting through its receptor 2 (VEGF-R2), is the main proangiogenic factor associated with adipogenesis. Adipose tissue inflammation can downregulate PPARγ expression and impair the normal adipogenesis process [7]. A reduction of adipose tissue capacity to undergo adipogenesis can lead to

adipocyte hypertrophy, ectopic fat deposition, inflammation and ER stress [4, 8, 9].

Diet can play a major role in the prevention of adiposity-associated pathologies. Flavonoids are the most abundant phenolic compounds in the plant kingdom. The ability of flavonoids to modulate cell signaling could contribute to the health benefits associated with consumption of fruits and vegetables [10]. Among flavonoids, the flavonol quercetin (Q) is one of the most abundant flavonoids in human dietary sources such as onions, broccoli, apples, blueberries, tea, grapes, and several other plants and derived products, and has been proposed to mitigate obesity-associated pathologies [11-13]. Q was shown to exert anti-inflammatory actions both *in vivo* and *in vitro*. We previously reported that Q downregulated pro-inflammatory signaling cascades (mitogen-activated kinases JNK and p38) and adipocytokine expression (resistin, MCP-1, TNFα) in the epididymal white adipose tissue (eWAT) from high fructose fed rats and in 3T3-L1 adipocytes [12]. Q also decreases adipose macrophage infiltration in mice fed a Western style diet [13, 14].

We previously showed that a grape pomace extract, rich in flavonoid compounds including Q, protects high-fat diet-induced adipose hypertrophy and dysfunction in rats [15]. Thus, identifying the active components responsible for the capacity of grape pomace extract to protect against diet-induced adipose dysfunction is relevant in order to generate diets that can mitigate obesity associated pathologies. In this study, we tested the hypothesis that Q can protect high-fat diet-induced adipose hypertrophy and inflammation promoting adipose tissue adipogenesis. The underlying mechanisms of action of Q and its

main metabolite quercetin 3-glucuronide during adipocyte differentiation were investigated in 3T3-L1 pre-adipocytes.

2. Materials and methods

2.1. Materials

Total cholesterol, HDL cholesterol and triglyceride assay kits were purchased from GTLab. (Buenos Aires, Argentina). Glucose was measured using a glucometer (Accu-Chek Performa, Roche, Buenos Aires, Argentina). The Ultra-Sensitive Insulin ELISA Kit was purchased from Crystal Chem (Downers Grove, IL, USA). The primary antibody for VEGF-A (MAB293) was from R & D Systems (Minneapolis, MN). Primary antibodies for adiponectin (C45B10) #2789, fatty acid synthase (C20G5) #3180, C/EBPα (D56F10) #8178 and PPARγ (C26H12) #2435 were obtained from Cell Signaling Technology (Danvers, MA). Primary antibodies for PPARγ (sc-7273), TLR-4 (sc-293072), HIF-1α (sc-13515), ATF-6 (sc-22799), XBP-1 (sc-32136), Flk-1/VEGF-R2 (sc-504), CD68 (sc-59103), MCP-1 (sc-1785), JNK (FL) sc-572 and p-JNK ((Thr183/Tyr185)) (sc-6254) were from Santa Cruz Biotechnology (Santa Cruz, CA). Nitrocellulose membranes were obtained from BIO-RAD (Hercules, CA). All other chemicals were purchased from Sigma Aldrich Co (St. Louis, MO).

Mouse embryo 3T3-L1 pre-adipocyte cells line were obtained from American Type Culture Collection, (Manassas, VA). Modified Medium from Dulbecco Eagle (DMEM) was obtained from ThermoFisher (11965-092), fetal bovine serum (FBS) from GIBCO (10437-028), dexamethasone from Sigma (#D-1756) and the insulin from Humulin Lilly (U-100 NDC 0002-8215-01 Hi-210).

2.2. Animals and animal care

All procedures were approved by the Institutional Animal Care and Use Committee of the School of Medical Science, University of Cuyo (Protocol approval N° 36/2014). Rats were housed under controlled conditions of temperature (23°C±1°C) and light (12 h light/12 h dark cycle). Eight week (w) old male Sprague Dawley rats were randomly divided (7 rats/group) into three experimental groups and fed for 6 w the following diets: (1) a standard chow diet (Control group) (Ctrl), (2) the chow diet containing 40% calories from fat (w/w) (20% bovine and 20% porcine fat) (HFD) and (3) the HFD supplemented with 20 mg Q/kg body weight (bw) (HFD+Q). The amount of Q was chosen based on our previous study [12] and on studies by other groups in rat models of obesity or hypertension [11, 16]. The fat and Q were incorporated into the pellet mixture as previously described [12, 15]. Every 2–3 days (d), pellets were weighed and dispensed. Body weight was recorded weekly. Food and water intakes were determined three times a w. After 6 w in the dietary treatments and after an overnight fast, rats were weighed and anesthetized with ketamine (50 mg/kg bw) and acepromazine (1 mg/kg bw), and blood was collected from the abdominal aorta into EDTA-containing tubes. Plasma was obtained after centrifugation at 1000×g for 15 min at 4°C. eWAT and subscapular brown adipose tissue (BAT) were collected and weighed. Tissues were flash frozen in liquid nitrogen and then stored at −80°C for further analysis. A piece of eWAT

was immediately fixed in 10% (w/v) neutral formalin solution for 24 h and processed for histological analysis.

2.3. Metabolic measurements

Glucose was measure in blood collected from the tail using a glucometer. For glucose tolerance tests (GTT), overnight fasted rats were injected with D-glucose (2 g/kg bw), and blood glucose was measured before and at 15, 30, 60, and 120 min post-injection as previously described [17, 18]. GTT was done after 4 w on the respective diets. At the end of the study, plasma total cholesterol, triglycerides, and HDL cholesterol concentrations were determined by enzymatic colorimetric methods using commercial kits. Insulin was measured using the Ultra-Sensitive Insulin ELISA kit and IR was evaluated using the homeostasis model assessment parameter (HOMA-IR) using the following formula: HOMA-IR (mg/dL × μU/mL) = fasting glucose (mg/dL) × insulin in fasting (μU/mL)/405. Plasma free fatty acids (FFA) and TNFα concentrations were determined using commercial kits (HR Series NEFA-HR, Fujifilm Wako Diagnostics, CA, USA and RayBiotech, Inc., Norcross, GA, USA, respectively). Assays were ran according to the manufacturer's protocol.

2.4. Western blots analysis

Tissue homogenates were prepared as previously described [12, 17]. Aliquots of total homogenates containing 25–40 μg protein were separated by reducing 8%–12.5% (w/v) polyacrylamide gel electrophoresis and electroblotted onto

nitrocellulose membranes. Membranes were blocked for 2 hours (h) in 5% (w/v) nonfat milk and subsequently incubated in the presence of the corresponding primary antibodies (1:1000 dilution) overnight at 4°C. After incubation for 90 min at room temperature in the presence of secondary antibodies (either HRP or biotinylated antibodies, followed in the later case by 1 h incubation with streptavidin), the conjugates were visualized and quantified by chemiluminescence detection in a Luminescent Analyzer Image Reader (LAS-4000) (Fujifilm, Japan). The densitometric analysis was performed using the Image J Program.

2.5. Histological analysis

Adipocyte hypertrophy degree was evaluated in eWAT sections prepared from paraffin-embedded samples and stained with hematoxylin and eosin. Images were analyzed using a CCD camera (Nikon, Japan) at 20× magnification, and adipocyte area was measured using the Image J program. Values are the average of three sections per animal (7 animals per group).

2.6. Immunofluorescence

Paraffin sections of eWAT were dewaxed and then hydrated. After a 5 min wash with PBS, samples were incubated overnight with 50 mM $ClNH_4$ under UV light. After 3 washes with PBS, samples were blocked with BSA in PBS (1mg/ml) for 2 h with gentle agitation at room temperature. Samples were washed and sections incubated with the primary antibody (dilution 1:50 in PBS) overnight at 4° C. Sections were subsequently washed and incubated with an anti-mouse secondary antibody conjugated with Cy3 (dilution 1: 400 in PBS) for

2 h at room temperature. After washing and drying, sections were mounted with Fluoroshield (Sigma-Aldrich, USA). Images were obtained in a confocal microscope-Olympus-FV-1000 (Japan).

2.7. Cell culture and incubations

Mouse embryo 3T3-L1 pre-adipocyte cells line were cultured in complete medium (CM) consisting of Modified Medium from Dulbecco Eagle (DMEM) rich in glucose supplemented with 10% fetal bovine serum (FBS) (v/v), 1% penicillin/streptomycin and 2 mM glutamine in an atmosphere of 5% (v/v) CO_2 at 37° C. After 2 d of confluence (d 0), differentiation was started by incubating the cells with induction medium: CM supplemented with 1M isobutylmethylxanthine, 1μM dexamethasone and 1mM insulin for 48 h, followed by CM supplemented with insulin for additional 48 h. At (d 0), cells were treated without or with 20 ng/mL TNFα, as previously described by Hammarstedt et al. [19], and in the absence or the presence of 1 and 10 μM Q (Q1 and Q10) or of the Q metabolite quercetin 3-glucuronide (G1 and G10), and were replaced with the change of culture media.

2.8. Statistical analysis

Data are shown as mean ± S.E.M. Statistical significances were assessed by one-way ANOVA followed by Bonferroni's Multiple Comparison Test. GraphPad Prism version 5.00 for Windows (Graph-Pad Software, San Diego, CA, USA) was used for all statistical analysis. A p value < 0.05 was considered statistically significant.

3. Results

3.1. Effect of Q on metabolic parameters in rats fed a high-fat diet

The effects of Q supplementation on metabolic parameters in rats fed for 6 w control, high-fat diet, or Q-supplemented high fat diets are shown in **Table 1.** Daily food intake was lower in the HFD and HFD+Q compared to the Ctrl group, although the average energy intake was similar for all groups. No statistically significant differences were observed in body weight, plasma glucose, TG, HDL, total cholesterol and FFA concentrations among groups. On the other hand, TNFα plasma concentration was increased in the HFD compared to the Ctrl group, while Q supplementation prevented high-fat diet-increased TNFα concentrations.

Rats fed the high-fat diet had lower ability to metabolize circulating glucose 30 min after performing the GTT when compared to the Ctrl group **(Fig. 1A)**. The GTT area under the curve (AUC) was significantly higher in the HFD compared to Ctrl and HFD+Q groups **(Fig. 1B).** High-fat diet consumption caused high levels of plasma fasting insulin and HOMA: IR values after 6 w on the diet. Both changes were prevented by Q supplementation **(Fig. 1C and D)**. Together, data indicate that Q supplementation attenuates high-fat diet-induced glucose intolerance, IR and inflammation.

3.2. Q increased brown adipose weight and prevented high-fat diet-induced adipocyte hypertrophy

Consumption of a high-fat diet significantly increased eWAT weight and adipocyte size compared to the Ctrl group. The addition of Q to the high-fat diet did not modify eWAT weight, but significantly reduced adipocyte size (Table 1 and Fig. 2A and B). In addition, in the HFD+Q group we observed a partial and

significant increase BAT weight compared to the HFD and Ctrl groups, respectively (Table 1). When the eWAT/BAT ratio was calculated, we observed a significantly increased eWAT/BAT ratio in HFD rats, which was prevented upon Q supplementation (Table 1). Overall, these results indicate that Q supplementation increased BAT mass and prevented high-fat diet-induced adipocyte hypertrophy in rats fed a high fat diet.

3.3. Effect of Q on HIF-1α, angiogenesis and adipogenesis in the eWAT from rats fed a high-fat diet

In order to explore whether Q could reduce the size of adipocytes, proteins involved in angiogenesis and adipogenesis were measured in eWAT by Western blot **(Fig. 3)**. Consumption of the high-fat diet increased HIF-1α protein levels and reduced those of VEGF-A and VEGF-R2. Q supplementation significantly reduced HIF-1α levels and increased both angiogenic factors, VEGF-A and VEGF-R2 **(Fig. 3.A)**.

In mammalian cells transcription factors PPARγ and C/EBPα, β and δ, are considered the main regulators of early adipogenesis, whereas adiponectin and fatty acid synthase (FAS) are responsible for mature adipocyte formation. Therefore, protein levels of PPARγ, C/EBPα, adiponectin and FAS were measured as parameters of adipogenesis in the eWAT **(Fig. 3.B)**. Chronic consumption of the high-fat diet caused downregulation of PPARγ, C/EBPα, adiponectin and FAS. All this changes were prevented by Q supplementation. The above results indicate that Q can mitigate high-fat diet-induced adipose hypertrophy and hypoxia in part by activating angiogenesis and adipogenesis.

3.4. Supplementation with Q attenuated high-fat diet-induced adipose tissue inflammation

Macrophages are crucial contributors to the inflammatory process at local (adipose tissue) and systemic levels. Together with ER stress, macrophages contribute to the development and perpetuation of adipose tissue inflammation induced by obesity **(Fig. 4).**

Western blot analysis showed that a high-fat diet consumption increased adipose tissue macrophage infiltration and inflammation, as evidenced by higher eWAT levels of TLR-4, CD68, MCP-1, and phosphorylated (Thr183/Tyr185) JNK, and ER stress markers as ATF-6 and XBP-1. Q supplementation attenuated high-fat diet-induced TLR-4 increase and reduced CD68, MCP-1, p-JNK and ER stress protein expression **(Fig. 4A and C).** The activation of TLR-4 in the HFD was confirmed by immunofluorescence in histological sections of eWAT **(Fig. 4B)**. These results confirm that consumption of a high-fat diet stimulates macrophage infiltration in the eWAT, as evidenced by a higher expression of the macrophage marker CD68, which was prevented by Q supplementation.

3.5. Effects of Q and quercetin 3-glucuronide on proteins involved in adipogenesis in 3T3-L1 pre-adipocytes treated with TNFα

In order to evaluate the effects of inflammation during early adipocyte differentiation, 3T3-L1 pre-adipocytes were induced to differentiate and simultaneously treated for 4 d with or without TNFα (20 ng/mL), and in the

absence or the presence of 1 and 10 μM Q (Q1 and Q10) or quercetin 3-glucuronide (G) (G1 and G10).

TNFα significantly reduced adipogenesis by approximately 50%, as evidenced by lower protein levels of PPARγ, C/EBPα, FAS and adiponectin compared to non-treated cells **(Fig. 5).** Incubation with Q 1-10 μM and G 10 μM significantly increased PPARγ levels compared to TNFα-treated cells (37, 39 and 38%, respectively). Q (10 μM) and G (1-10 μM) significantly increased C/EBPα, while both concentrations of Q and G increased FAS protein levels, to values of control non-treated cells. In addition, Q (1-10 μM) and G at 1 μM significantly increased adiponectin levels compared to TNFα treated cells **(Fig. 5).** Overall, Q and G attenuated TNFα-impaired adipogenesis in pre-adipocyte 3T3-L1 cells.

4. Discussion

Chronic consumption of a high-fat diet increased adiposity, adipose hypertrophy, and inflammation in rats, leading to the development of IR. Simultaneous consumption of Q significantly reduced the adipocyte size, attenuated adipose inflammation, and prevented systemic IR. These effects can be attributed, in part, to the capacity of Q to stimulate a healthy adipose tissue expansion promoting adipose angiogenesis and adipogenesis.

As a consequence of a continuous excess of energy availability, adipose tissue vascularization may be insufficient to maintain tissue normoxia. Under hypoxic conditions, adipose tissue increases the synthesis of inflammatory cytokines, in part due to HIF-1 activation, contributing to increased macrophage infiltration and inflammation [20]. In addition, the type of dietary fats (saturated fats) as well as an increase in circulating free fatty acids stimulates the activation of TLR-4 present in different cells such as macrophages. TLR-4 signaling induces

the transcription of several genes involved in the inflammatory response, such as pro-inflammatory cytokines, and the recruitment of pro-inflammatory macrophages, contributing to low-grade chronic inflammation and IR [5]. Accordingly, we currently observed that the presence of hypertrophic and hypoxic adipocytes in the eWAT of the HFD group were accompanied with an increased expression of TLR-4, CD68 (parameter of macrophage infiltration), the chemokine MCP-1 and the pro-inflammatory kinase JNK. Activation of this pathways leads to the transcription of pro-inflammatory cytokines that contribute to a cycle of chronic inflammation. In agreement, the HFD group was accompanied with increased TNFα plasma concentration.

Given the secretory capacity of adipose tissue, the metabolic stress induced by excess nutrients and hypertrophy can lead to oxidative stress and accumulation of missfolded proteins in the lumen of the ER causing dysfunction of organelles, in particular of mitochondria and the ER. ER stress regulates adipocytes inflammation in obese rats through TLR-4 signaling [4]. In this sense, the reduction of adipose tissue and the metabolic improvement in obese patients undergoing bariatric surgery is associated with a downregulation of ER stress evidenced by a decrease in JNK and XBP-1 activation [21]. We observed that Q mitigated TLR-4 activation and the expression of CD68, MCP-1 and ER stress associated with fat consumption, together with a reduction of TNFα plasma concentration, while increased the anti-inflammatory and insulin sensitive cytokine (adiponectin). These data suggest that Q could counteract high fat diet-induced macrophage infiltration and inflammation in the adipose tissue. In agreement, Q suppressed lipopolysaccharides induced-TLR-4 signaling in

macrophages 264.7 through the positive regulation of Tollip expression (a negative regulator of TLR signaling) [22].

It is known that adipose hypertrophy promotes tissue inflammation and dysfunction, while a reduction of adipocyte diameter, even in the absence of adipose tissue mass decrease, may prevent these alterations [3]. Accordingly, chronic consumption of a high-fat diet increased eWAT weight and caused adipocyte hypertrophy while supplementation with Q reduced adipose hypertrophy, without modifying the eWAT weight. These can be in part attributed to the capacity of Q to prevent high-fat diet-mediated increase in adipose protein levels of HIF-1α and proteins that participate in angiogenesis and adipogenesis [23, 24]. These findings also suggest that Q promotes hyperplasia instead of hypertrophy. However, considering the increased BAT weight and the decreased eWAT/BAT ratio observed in the HFD+Q we could also assume an increase in energy expenditure. Consistently, we previously showed that Q up-regulates proteins involved in mitochondrial biogenesis in 3T3-L1 adipocytes treated with palmitate [15]. In addition, Q promotes WAT brown-like adipocyte appearance in mice and in 3T3-L1 cells [25, 26]. Therefore, we cannot discard a possible increase of BAT thermogenesis and/or browning effect on WAT.

There are controversial and contradictory results in the literature based on the inhibitory effects of polyphenols, including Q, on adipogenesis and angiogenesis. Q suppressed adipogenesis by reducing the expression of C/EBPα, PPARγ and inflammatory cytokines in 3T3-L1 cells and in a mouse model of obesity [27]. Q also suppressed lipogenesis by reducing the rate of fatty acid incorporation into triglycerides in adipocytes, by decreasing the

expression of FAS and acetyl CoA-carboxylase [28]. Q also inhibits PPARγ and C/EBPα expression in 3T3-L1 pre-adipocytes at very high concentrations (50-100 μM), while at lower concentrations (10 μM) the opposite effect was observed [28]. In addition, Q (10 μM) promoted adipogenesis in human mesenchymal stem cells [24]. Many authors suggest that adipose tissue angiogenesis and/or adipogenesis inhibition could be a strategy to prevent adipose tissue growth and obesity. However, an impairment of adipogenesis may lead to hypertrophic adipocytes, increased ectopic fat deposition, and IR [29]. In line with this, obese mice that overexpress adiponectin showed higher expression of PPARγ in the adipose tissue, increased in adipocyte cell number and adipose tissue mass, had lower macrophage infiltration, and reduced systemic inflammation with normalized metabolic parameters [30]. We have previously shown that Q attenuates/prevents high fructose or TNFα-induced expression of pro-inflammatory adipocytokines (visfatin, resistin and MCP-1) and increases the expression of adiponectin in the visceral adipose tissue and in 3T3-L1 adipocytes, respectively [12]. In addition, incubation with TNFα (50 ng/mL) for 24 h decrease the transcription of the PPARγ gene and the expression of C/EBPδ and its binding to DNA in 3T3-L1 pre-adipocytes during early adipocytes differentiation [7], which suggest that inflammation could be a key factor of early interruption of adipogenesis associated with increased IR [31]. Accordingly, in this study we observed that Q and quercetin 3-glucuronide attenuated TNFα-induced alterations in proteins involved in early adipocyte differentiation in 3T3-L1 pre-adipocytes (PPARγ and C/EBPα, and key proteins responsible for the maturation of adipocytes (FAS and adiponectin).

In summary, the capacity of Q to modulate: i) adipose tissue hypertrophy (decreased adipose diameter), ii) the consequent activation of the redox sensitive pathways JNK and TLR-4, and iii) ER stress; would converge in the mitigation of the inflammation-induced IR associated to consumption of a high-fat diet in rats. These findings support the potential relevance of consuming Q-rich foods or supplements to attenuate high fat diet-induced adipose dysfunction and IR.

Funding: This work was supported by the Universidad Nacional de Cuyo [Programa I+D 2015, SeCTyP, SIIP], Agencia Nacional de Promoción Científica y Tecnológica [PICT 2014-0547, PICTO 2016-0046, PICT 2018-03056] to M.V.P, and NIFA-USDA (CA-D*-XXX-7244-H) to P.O.

Table 1. Q supplementation effects on metabolic parameters in rats with a high-fat diet.

Parameter	Ctrl	HFD	HFD+Q
Food intake (g)	28.5 ± 1.4[a]	18.7 ± 0.9[b]	19.3 ± 1.2[b]
Energy intake (Kcal/d)	89.7 ± 6.0	88.1 ± 7.4	91.0 ± 7.9
Body weight (BW) (g)	450.0 ± 12.9	478.6 ± 10.3	459.0 ± 22.6
Fasted glucose (mg/dl)	100.0 ± 1.9	109.0 ± 2.3	103.0 ± 3.3
eWAT (mg)/BW	805.0 ± 33.9[a]	1628.0 ± 117.0[b]	1311.0 ± 122.0[b]
BAT (mg)/BW	98.4 ± 5.5[a]	121.0 ± 13.6[a,b]	150.0 ± 11.1[b]
eWAT/BAT	9.0 ± 1.0[a]	13.6 ± 1.1[b]	7.2 ± 1.0[a]
TG (mg/dl)	55.8 ± 8.4	64.0 ± 4.7	73.4 ± 0.8
Total cholesterol (mg/dl)	70.3 ± 4.9	63.8 ± 3.3	64.8 ± 7.7
HDL cholesterol (mg/dl)	33.6 ± 0.8	30.2 ± 1.3	31.30 ± 2.3
FFA (mEq/L)	1.5 ± 0.1	1.6 ± 0.1	1.5 ± 0.1
TNFα (pg/ml)	5.4 ± 0.3[a]	6.9 ± 0.4[b]	5.8 ± 0.2 [a]

Food intake and metabolic parameters of rats fed a control (Ctrl) or a high-fat diet without (HFD) or with supplementation with Q (20 mg/kg body weight/d)

(HFD+Q). Results are shown as the mean ± SEM of seven animals/treatment. Values having different superscripts are significantly different ($p < 0.05$, one-way ANOVA).

Legend to Figures:

Figure 1. Effects of Q supplementation on metabolic parameters in rats fed a high-fat diet. (A-D) Rats were fed a control (Ctrl) (white bars) or a high-fat diet without (HFD) (black bars) or with supplementation with Q (20 mg/kg body weight/d) (HFD+Q) (grey bars) for 6 weeks. (A) Glucose tolerance test (GTT) was done after 4 weeks on the respective diets, (B) area under the curve for the GTT, (C) plasma insulin concentration and (D) HOMA:IR. Results are shown as mean ± SEM of seven animals/group. Values having different superscripts are significantly different ($p < 0.05$, one-way ANOVA).

Figure 2. Effect of dietary Q supplementation on adipocyte area in eWAT from rats fed a high-fat diet. Rats were fed a control (Ctrl) (white bars) or a high-fat diet without (HFD) (black bars) or with supplementation with Q (20 mg/kg body weight/d) (HFD+Q) (grey bars) for 6 weeks. (A) Representative histological images of eWAT stained with hematoxylin and eosin (scale 50 µm), and (B) mean adipocyte diameter in eWAT. Results are shown as mean ± S.E.M. of seven animals/group. Values having different superscripts are significantly different ($p < 0.05$, one-way ANOVA).

Figure 3. Effect of dietary Q supplementation on proteins involved in hypoxia, angiogenesis and adipogenesis in the eWAT of rats fed a high-fat diet. Rats were fed a control (Ctrl) or a high-fat diet without (HFD) or with supplementation with Q (20 mg/kg body weight/d) (HFD+Q) for 6 weeks. (A) HIF-1α, VEGF-A and VEGF-R2, and (B) PPARγ, C/EBPα, adiponectin and FAS protein levels in the eWAT were measured by Western blot. Bands were quantified and values normalized to β-actin levels (loading control). Results were referred to control group values (Ctrl). Results are shown as the mean ± SEM of seven animals/group. Values having different superscripts are significantly different ($p < 0.05$, one-way ANOVA).

Figure 4. Q attenuated adipose tissue inflammation in rats fed a high-fat diet. Rats were fed a control (Ctrl) or a high-fat diet without (HFD) or with supplementation with Q (20 mg/kg body weight/d) (HFD+Q) for 6 weeks. (A) Protein levels of the inflammatory parameters TLR-4, CD68 and MCP-1 were measured by Western blot, (B) representative images of TLR-4 determined by immunofluorescence in sections of eWAT. From top to bottom: Ctrl, HFD and HFD+Q. Histological sections were examined by light field microscopy (right) and fluorescence (left) (scale 5 μm). Color bars indicate greater intensity at a greater number. Magnified areas are indicated with a box, (C) p-JNK, JNK, ATF-6 and XBP-1 protein levels in the eWAT were measured by Western blot. (A, C) Bands were quantified and values referred to β-actin levels (loading control), except for p-JNK that was normalized to total JNK levels. Results were referred to control group values (Ctrl). Results are shown as the mean ± SEM of

five animals/group. Values having different superscripts are significantly different ($p < 0.05$, one-way ANOVA).

Figure 5. Effect of Q and quercetin 3-glucuronide on adipogenic factors in 3T3-L1 pre-adipocytes. 3T3-L1 pre-adipocytes were incubated with or without Q and G (1-10 μM) for 4 d, and in the absence or the presence of TNFα (20 ng/mL). Protein levels of PPARγ, C/EBPα, FAS and adiponectin were determined by Western blot. Western blot bands were quantified and values normalized to β-actin levels (loading control). Results were referred to control group values (Ctrl). Results are shown as mean ± SEM of 3 independent experiments. Values having different superscripts are significantly different ($p<0.05$, one way ANOVA test).

References

1. Williams, E.P., et al., *Overweight and Obesity: Prevalence, Consequences, and Causes of a Growing Public Health Problem.* Curr Obes Rep, 2015. **4**(3): p. 363-70.
2. Engin, A., *The Definition and Prevalence of Obesity and Metabolic Syndrome.* Adv Exp Med Biol, 2017. **960**: p. 1-17.
3. Kalupahana, N.S., N. Moustaid-Moussa, and K.J. Claycombe, *Immunity as a link between obesity and insulin resistance.* Mol Aspects Med, 2012. **33**(1): p. 26-34.
4. Li, X., *Endoplasmic reticulum stress regulates inflammation in adipocyte of obese rats via toll-like receptors 4 signaling.* Iran J Basic Med Sci, 2018. **21**(5): p. 502-507.
5. Maury, E. and S.M. Brichard, *Adipokine dysregulation, adipose tissue inflammation and metabolic syndrome.* Mol Cell Endocrinol, 2010. **314**(1): p. 1-16.
6. Cawthorn, W.P., et al., *Tumour necrosis factor-alpha inhibits adipogenesis via a beta-catenin/TCF4(TCF7L2)-dependent pathway.* Cell Death Differ, 2007. **14**(7): p. 1361-73.
7. Kudo, M., et al., *Transcription suppression of peroxisome proliferator-activated receptor gamma2 gene expression by tumor necrosis factor alpha via an inhibition of CCAAT/ enhancer-binding protein delta during the early stage of adipocyte differentiation.* Endocrinology, 2004. **145**(11): p. 4948-56.
8. Bettaieb, A., et al., *Anti-inflammatory actions of (-)-epicatechin in the adipose tissue of obese mice.* Int J Biochem Cell Biol, 2016. **81**(Pt B): p. 383-392.
9. McQuaid, S.E., et al., *Downregulation of adipose tissue fatty acid trafficking in obesity: a driver for ectopic fat deposition?* Diabetes, 2011. **60**(1): p. 47-55.

10. Fraga, C.G., et al., *Basic biochemical mechanisms behind the health benefits of polyphenols.* Mol Aspects Med, 2010. **31**(6): p. 435-45.
11. Rivera, L., et al., *Quercetin ameliorates metabolic syndrome and improves the inflammatory status in obese Zucker rats.* Obesity (Silver Spring), 2008. **16**(9): p. 2081-7.
12. Vazquez Prieto, M.A., et al., *Catechin and quercetin attenuate adipose inflammation in fructose-fed rats and 3T3-L1 adipocytes.* Mol Nutr Food Res, 2015. **59**(4): p. 622-33.
13. Dong, J., et al., *Quercetin reduces obesity-associated ATM infiltration and inflammation in mice: a mechanism including AMPKalpha1/SIRT1.* J Lipid Res, 2014. **55**(3): p. 363-74.
14. Kobori, M., et al., *Quercetin suppresses immune cell accumulation and improves mitochondrial gene expression in adipose tissue of diet-induced obese mice.* Mol Nutr Food Res, 2016. **60**(2): p. 300-12.
15. Rodriguez Lanzi, C., et al., *Grape pomace extract induced beige cells in white adipose tissue from rats and in 3T3-L1 adipocytes.* J Nutr Biochem, 2018. **56**: p. 224-233.
16. Perez-Vizcaino, F., et al., *Antihypertensive effects of the flavonoid quercetin.* Pharmacol Rep, 2009. **61**(1): p. 67-75.
17. Bettaieb, A., et al., *(-)-Epicatechin mitigates high-fructose-associated insulin resistance by modulating redox signaling and endoplasmic reticulum stress.* Free Radic Biol Med, 2014. **72**: p. 247-56.
18. Rodriguez Lanzi, C., et al., *Grape pomace and grape pomace extract improve insulin signaling in high-fat-fructose fed rat-induced metabolic syndrome.* Food Funct, 2016. **7**(3): p. 1544-53.
19. Hammarstedt, A., et al., *Wnt-signaling is maintained and adipogenesis inhibited by TNFalpha but not MCP-1 and resistin.* Biochem Biophys Res Commun, 2007. **357**(3): p. 700-6.
20. Bluher, M., *Adipose tissue dysfunction in obesity.* Exp Clin Endocrinol Diabetes, 2009. **117**(6): p. 241-50.
21. Gregor, M.F., et al., *Endoplasmic reticulum stress is reduced in tissues of obese subjects after weight loss.* Diabetes, 2009. **58**(3): p. 693-700.
22. Byun, E.B., et al., *Quercetin negatively regulates TLR4 signaling induced by lipopolysaccharide through Tollip expression.* Biochem Biophys Res Commun, 2013. **431**(4): p. 698-705.
23. Sung, H.K., et al., *Adipose vascular endothelial growth factor regulates metabolic homeostasis through angiogenesis.* Cell Metab, 2013. **17**(1): p. 61-72.
24. Casado-Diaz, A., et al., *Effects of quercetin, a natural phenolic compound, in the differentiation of human mesenchymal stem cells (MSC) into adipocytes and osteoblasts.* J Nutr Biochem, 2016. **32**: p. 151-62.
25. Lee, S.G., J.S. Parks, and H.W. Kang, *Quercetin, a functional compound of onion peel, remodels white adipocytes to brown-like adipocytes.* J Nutr Biochem, 2017. **42**: p. 62-71.
26. Choi, H., C.S. Kim, and R. Yu, *Quercetin Upregulates Uncoupling Protein 1 in White/Brown Adipose Tissues through Sympathetic Stimulation.* J Obes Metab Syndr, 2018. **27**(2): p. 102-109.
27. Seo, M.J., et al., *The inhibitory effects of quercetin on obesity and obesity-induced inflammation by regulation of MAPK signaling.* J Nutr Biochem, 2015. **26**(11): p. 1308-16.
28. Ahn, J., et al., *The anti-obesity effect of quercetin is mediated by the AMPK and MAPK signaling pathways.* Biochem Biophys Res Commun, 2008. **373**(4): p. 545-9.
29. Dubois, S.G., et al., *Decreased expression of adipogenic genes in obese subjects with type 2 diabetes.* Obesity (Silver Spring), 2006. **14**(9): p. 1543-52.
30. Kim, J.Y., et al., *Obesity-associated improvements in metabolic profile through expansion of adipose tissue.* J Clin Invest, 2007. **117**(9): p. 2621-37.

31. Moseti, D., A. Regassa, and W.K. Kim, *Molecular Regulation of Adipogenesis and Potential Anti-Adipogenic Bioactive Molecules.* Int J Mol Sci, 2016. **17**(1).

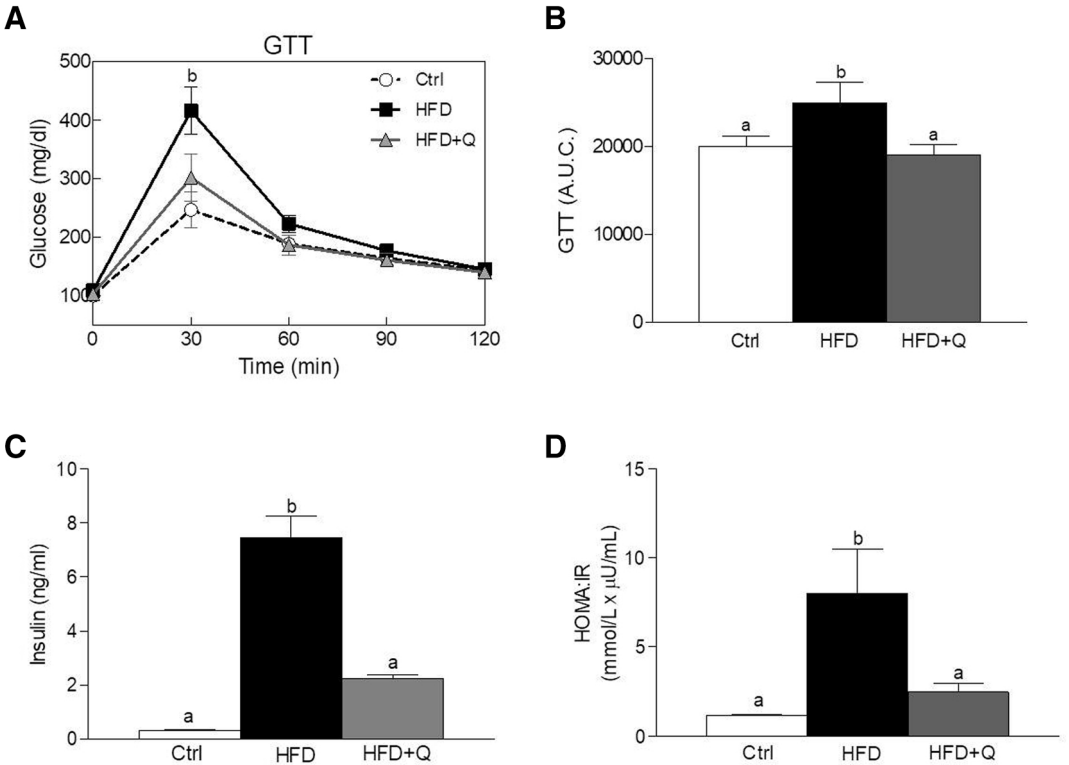

A
GTT
Ctrl
HFD
HFD+Q
Glucose (mg/dl)
Time (min)
B
GTT (A.U.C.)
C
Insulin (ng/ml)
D
HOMA:IR
(mmol/L x µU/mL)

Figure 1

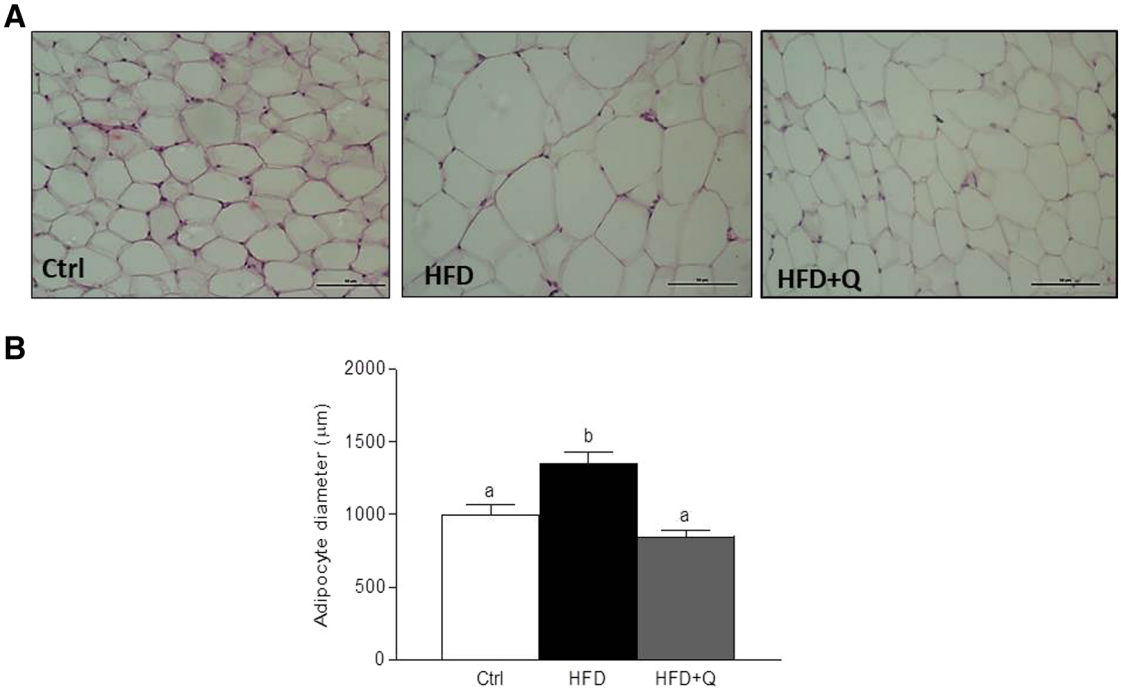

A
Ctrl
HFD
HFD+Q
B
Adipocyte diameter (µm)
2000
1500
1000
500
0
a
b
a
Ctrl
HFD
HFD+Q

Figure 2

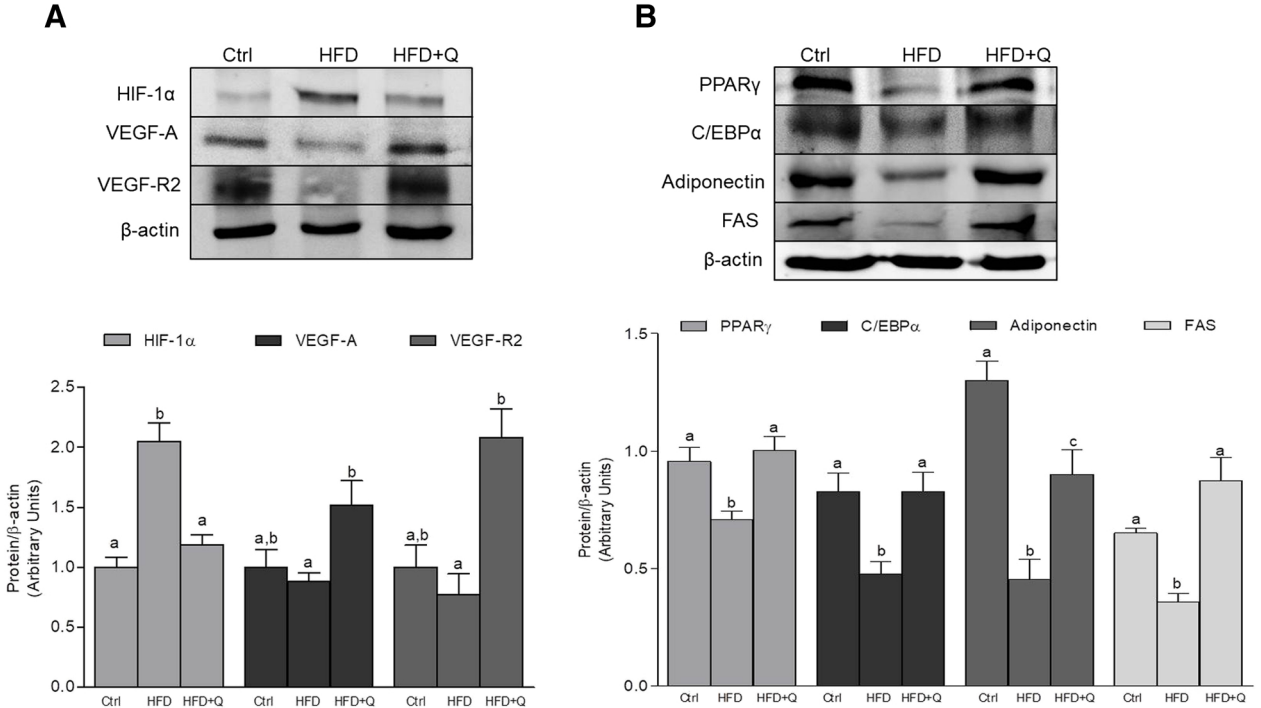

A
Ctrl
HFD
HFD+Q
HIF-1α
VEGF-A
VEGF-R2
β-actin
HIF-1α
VEGF-A
VEGF-R2
Protein/β-actin (Arbitrary Units)
B
Ctrl
HFD
HFD+Q
PPARγ
C/EBPα
Adiponectin
FAS
β-actin
PPARγ
C/EBPα
Adiponectin
FAS
Protein/β-actin (Arbitrary Units)

Figure 3

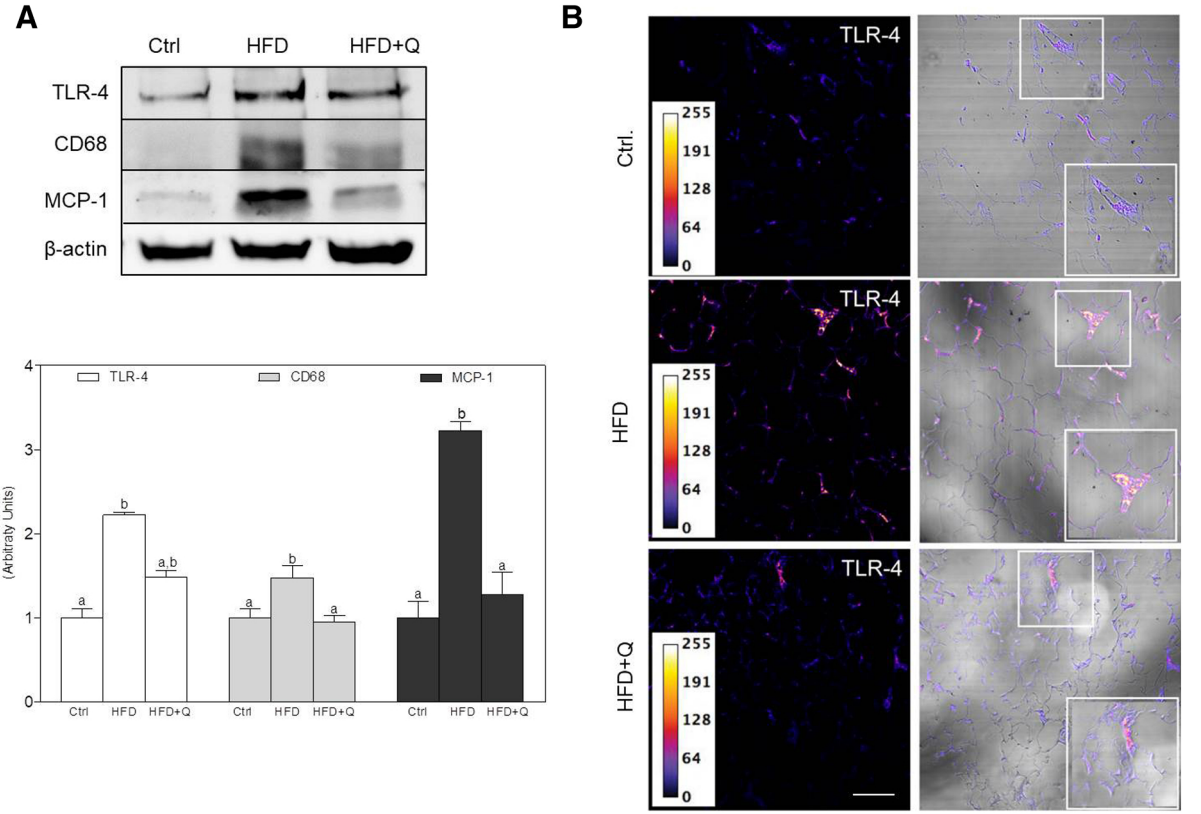

A
Ctrl
HFD
HFD+Q
TLR-4
CD68
MCP-1
β-actin
TLR-4
CD68
MCP-1
Protein/β-actin (Arbitraty Units)
0
1
2
3
4
a
b
a,b
Ctrl
HFD
HFD+Q
B
Ctrl.
HFD
HFD+Q
TLR-4
255
191
128
64
0

Figure 4ab

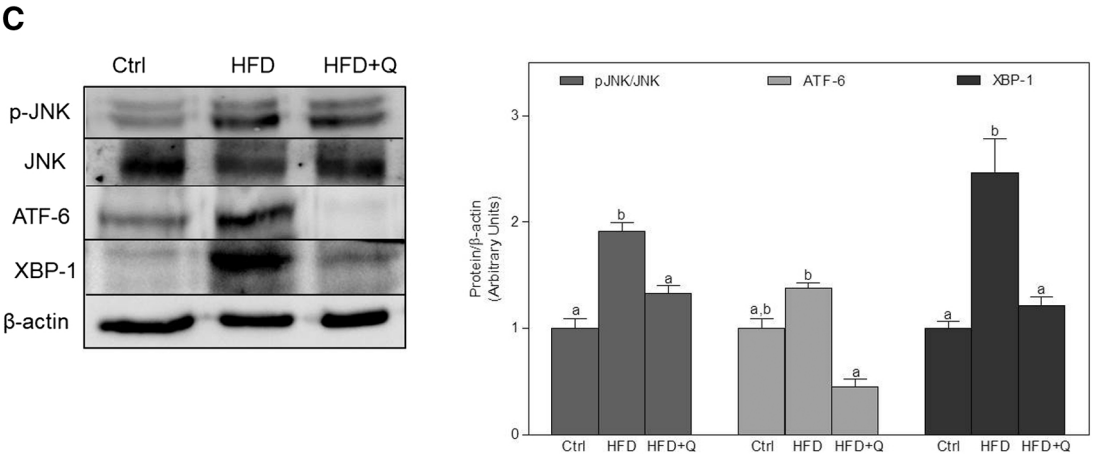
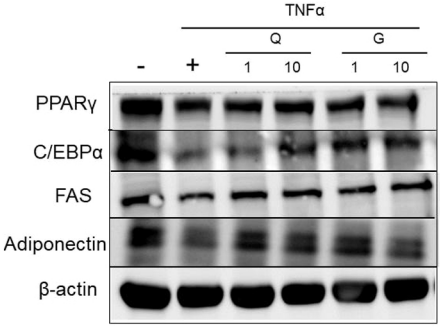

C
Ctrl
HFD
HFD+Q
p-JNK
JNK
ATF-6
XBP-1
β-actin
pJNK/JNK
ATF-6
XBP-1
Protein/β-actin
(Arbitrary Units)
0
1
2
3
a
b
a
a,b
b
a
a
b
a
Ctrl
HFD
HFD+Q
Ctrl
HFD
HFD+Q
Ctrl
HFD
HFD+Q

Figure 4c


TNFα
Q
G
-
+
1
10
1
10
PPARγ
C/EBPα
FAS
Adiponectin
β-actin

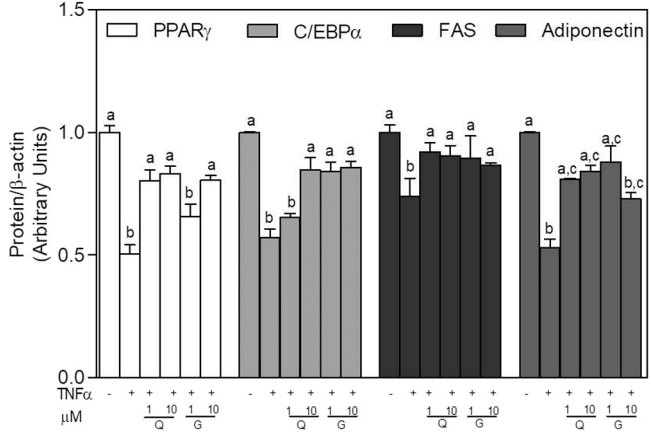

PPARγ
C/EBPα
FAS
Adiponectin
Protein/β-actin (Arbitrary Units)
1.5
1.0
0.5
0.0
TNFα
μM
Q
G

Figure 5