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Quercetin metabolites from *Hibiscus sabdariffa* contribute to alleviate glucolipotoxicity-induced metabolic stress *in vitro*

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ABSTRACT

Polyphenols from *Hibiscus sabdariffa* (HS) alleviate obesity-related metabolic complications but the metabolites responsible for such effects are unknown. We aimed to elucidate which of the potential plasma metabolites from a polyphenol-enriched HS (PEHS) extract contributed for the reversion of glucolipotoxicity-induced metabolic stress using 3T3-L1 adipocyte and INS 832/13 pancreatic β-cell models under glucolipotoxic conditions.

PEHS extract, quercetin (Q) and quercetin-3-O-glucuronide (Q3GA) showed stronger capacity to decrease glucolipotoxicity-induced ROS generation than ascorbic acid or chlorogenic acid. PEHS extract, Q and Q3GA decreased secretion of cytokines (leptin, TNF-α, IGF-1, IL-6, VEGF, IL-1α, IL-1β and CCL2) and reduced CCL2 expression at transcriptional level. In addition, PEHS extract, Q and Q3GA reduced triglyceride accumulation, which occurred through fatty acid synthase (FASN) downregulation, AMPK activation and mitochondrial mass and biogenesis restoration via PPARα upregulation. Electron microscopy confirmed that PEHS extract and Q3GA decreased mitochondrial remodeling and mitophagy. Virtual screening leads us to postulate that Q and Q3GA might act as agonists of these protein targets at specific sites.

These data suggest that Q and Q3GA may be the main responsible compounds for the capacity of PEHS extract to revert glucolipotoxicity-induced metabolic stress through AMPK-mediated decrease in fat storage and increase in fatty acid oxidation, though other compounds of the extract may contribute to this capacity.

1. Introduction

Adipocytes are far from being an inert fat storage tissue. Recent evidence supports the idea that these cells play an instrumental role in glucose homeostasis by regulating the presence of glucose transporter type 4 (GLUT4) receptors on the cell surface. Excess circulating glucose can be incorporated and converted to fatty acids through the activation of the lipogenic pathway. Fatty acids can then be stored in the form of triglycerides in lipid droplets. However, lipid overload generates endoplasmic reticulum stress, mitochondrial dysfunction and increased inflammatory cytokine release from adipocytes, attracting macrophages. This glucolipotoxicity is associated with an oxidative-inflammatory stress condition that, when sustained over time, results in insulin resistance in peripheral cells, mainly hepatocytes and myocytes (Mittra et al., 2008). Additionally, adipocytes become insulin resistant, decreasing glucose uptake and leading to hyperglycemia and the development of type 2 diabetes.

Obesity, considered as an excessive fat accumulation, is a risk condition that favors the development of type 2 diabetes and cardiovascular diseases and is associated with the diagnostic features of metabolic syndrome (Despres and Lemieux, 2006). One-quarter of the world's

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Abbreviations

ACC	acetyl-CoA carboxylase
AMPK	AMP-activated protein kinase
CCL2	chemokine (C–C motif) ligand 2
FASN	fatty acid synthase
FBS	fetal bovine serum
H_2DCF-DA	2′,7′-dichlorodihydrofluorescein diacetate
HS	*Hibiscus sabdariffa* L.
IBMX	3-isobutyl-1-methylxanthine
IGF-1	insulin-like growth factor-1
IL-1α	interleukin-1 alpha
IL-1β	interleukin-1 beta
IL-6	interleukin-6
pAMPK	phospho-AMPK
PEHS	polyphenol-enriched HS
PPARα	peroxisome proliferator-activated receptor alpha
Q	quercetin
Q3GA	quercetin-3-O-glucuronide
ROS	reactive oxygen species
TNF-α	tumor necrosis factor-alpha
VEGF	vascular endothelial growth factor

population suffers from metabolic syndrome, according to International Diabetes Federation (O'Neill and O'Driscoll, 2015). As mentioned before, excess fat accumulation compromises cellular function through severe defects in mitochondrial biogenesis and impaired mitochondrial dynamics, resulting in the overproduction of reactive oxygen species (ROS), which leads to oxidative stress (Addabbo et al., 2009). Since mitochondria are the main site for fat oxidation, mitochondrial dysfunction results in fat accumulation and adipocyte hypertrophy. In this context, the stimulation of the secretion of proinflammatory cytokines, such as interleukin (IL)-1, IL-6, tumor necrosis factor alpha (TNF-α), leptin and chemokine (C–C motif) ligand 2 (CCL2), may contribute to the development of insulin resistance (Cancello and Clement, 2006; Han, 2016; Luna-Luna et al., 2015; O'Neill and O'Driscoll, 2015).

Obesity-related dyslipidemia affects other tissues, such as the pancreas, leading to a glucolipotoxic condition that impairs pancreas endocrine function (Miyake et al., 2018). Pancreatic β-cells are located in the endocrine pancreas and secrete insulin. Fat accumulation in this particular cell type inhibits insulin secretion and results in mitochondrial dysfunction, leading to apoptosis. Low insulin production and secretion causes hyperglycemia and dyslipidemia, ultimately culminating in type 2 diabetes (Maestre et al., 2003). In fact, both insulin resistance in adipocytes and the dysfunction of pancreatic β-cells have been reported to be involved in the pathogenesis of metabolic syndrome. Thus, mitochondrial dysfunction might be regarded as a target for therapeutic action in metabolic diseases (Bhatti et al., 2017).

Polyphenols from *Hibiscus sabdariffa* L. (HS) have been shown to inhibit adipogenesis in 3T3-L1 preadipocytes (Kim et al., 2007), reduce body weight gain and glycemia in obese/MSG-treated mice (Alarcon-Aguilar et al., 2007) and decrease plasma triglycerides in hyperlipidemic mice (Fernandez-Arroyo et al., 2011). Furthermore, HS extract has been demonstrated to reduce the plasma concentrations of CCL2 in healthy humans (Beltran-Debon et al., 2010) and alleviates steatohepatitis in hyperlipidemic mice (Joven et al., 2012) and obese humans (Chang et al., 2014). These findings suggest that the polyphenolic extract of HS may modulate pathways related to energy management and inflammation, improving metabolic disturbances associated with obesity.

Extracts derived from HS calyces show a complex composition and are rich in anthocyanins (such as delphinidin and cyanidin derivatives) and polysaccharides (Herranz-Lopez et al., 2012; Rodriguez-Medina et al., 2009). HS extract also contains organic acids such as hydroxycitric and hibiscus acids, phenolic acids and other flavonoids such as quercetin (Q), luteolin and their derivatives (Herranz-Lopez et al., 2017b). We have previously shown that a polyphenol-enriched HS (PEHS) extract devoid of fiber and polysaccharides exerted a higher capacity than the original aqueous extract to inhibit triglyceride accumulation in hypertrophic and insulin-resistant 3T3-L1 adipocytes (Herranz-Lopez et al., 2012). PEHS extract also modulated proinflammatory adipokine secretion and reduced ROS generation in hypertrophic cells (Herranz-Lopez et al., 2012). Nevertheless, attempts to increase this activity by further purification failed, suggesting putative synergistic interactions between several components in the extract.

The experiments aimed at searching for the key compounds responsible for the properties mentioned above are scarce. A bioavailability study of PEHS extract in a rat model revealed that some flavonol-conjugated forms may be candidate metabolites that contribute to the health effects of PEHS extract, and Q and quercetin-3-O-glucuronide (Q3GA) were predominant metabolites in rat plasma with high elimination half-life values (Fernandez-Arroyo et al., 2012). Compared with other alternative plant sources (Nemeth and Piskula, 2007), PEHS extract has shown greater bioavailability of its quercetin derivatives, displaying a plasma concentration near 8 μM after oral administration in rats (Fernandez-Arroyo et al., 2012). Accordingly, the Q3GA metabolite was identified in the liver of hyperlipidemic mice fed PEHS extract, concomitantly with steatohepatitis improvements (Joven et al., 2012).

In view of the above, further research is needed to identify the specific metabolites responsible for the health effects of PEHS extract to develop polyphenolic mixtures from HS with potential applications in obesity. To identify these metabolites, in this work, we studied the effects of PEHS extract and some of the putative candidate metabolites in obesity-associated metabolic disturbances in 3T3-L1 hypertrophic cells and INS 832/13 pancreatic β-cells under glucolipotoxic conditions. The capacity of these metabolites to activate AMP-activated protein kinase (AMPK) and to modulate lipid metabolism and mitochondrial function was also evaluated. Finally, their putative ability to interact with selected target proteins related to energy management was also assessed using virtual screening.

2. Materials and methods

2.1. Chemicals and reagents

Dulbecco's modified Eagle's medium (DMEM), Roswell Park Memorial Institute medium (RPMI 1640), Dulbecco's phosphate-buffered saline (PBS), penicillin-streptomycin and D-glucose were purchased from Gibco (Grand Island, NY, USA). Dexamethasone, 3-isobutyl-1-methylxanthine (IBMX), insulin, sodium pyruvate, palmitic acid, β-mercaptoethanol, dimethyl sulfoxide (DMSO) and 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) were obtained from Sigma-Aldrich (Madrid, Spain). Calf serum and fetal bovine serum (FBS) were purchased from Thermo Scientific (Cramlington, Northumberland, UK). Fatty acid-poor and endotoxin-free fraction V albumin from bovine serum was obtained from EMD Millipore (Billerica, USA). PEHS extract was kindly provided by the Biomedical Research Unit (URB) (Reus, Spain) (Herranz-Lopez et al., 2012). Q was obtained from Extrasynthese (Genay, France). Q3GA, chlorogenic acid and L-ascorbic acid were purchased from Sigma-Aldrich. Cellulose acetate filters (0.2 μm) were obtained from GE Healthcare Life Sciences (Buckinghamshire, UK). AdipoRed™ Assay Reagent was purchased from Lonza (Walkersville, MD, USA).

2.2. Cell culture, differentiation of the 3T3-L1 cell line and hypertrophic adipocyte model

The 3T3-L1 preadipocytes (American Type Culture Collection,

Manassas, VA, USA) were cultured in low glucose (5 mM) DMEM supplemented with 10% calf serum, 100 μg/mL streptomycin and 100 U/mL penicillin. INS 832/13 pancreatic β-cells (C.B. Newgard, Duke University, Durham NC) were cultured in RPMI 1640 supplemented with 11 mM glucose, 10 mM HEPES, 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate and 50 μM β-mercaptoethanol as previously indicated (Maestre et al., 2003). Both cell lines were incubated at 37 °C in a humidified (5% CO_2, 95% air) atmosphere.

The 3T3-L1 preadipocytes were differentiated using standard protocols (Green and Kehinde, 1975). Briefly, differentiation from preadipocytes to adipocytes was induced by adding 10% FBS, 1 μM insulin, 1 μM dexamethasone and 0.5 mM IBMX to high glucose (25 mM) DMEM for 48 h. Then, the cells were maintained in high glucose DMEM supplemented with FBS and insulin, and medium was replaced every 2–3 days; mature adipocytes were obtained after 8 days of incubation. Hypertrophic adipocytes were obtained by maintaining mature adipocytes in high glucose DMEM supplemented with FBS and insulin for at least 9 additional days. Once hypertrophic adipocytes were obtained, the cells were treated with PEHS extract and several pure compounds for 48 h (namely ascorbic acid, chlorogenic acid, Q and Q3GA). Before the cell treatments, the extract and all the compounds were dissolved in medium or DMSO-medium and filtered for sterilization.

2.3. Induction of glucolipotoxicity

Glucolipotoxicity was induced in INS 832/13 pancreatic β-cells and mature 3T3-L1 adipocytes by incubation in 25 mM glucose alone or 25 mM glucose plus 0.5 mM palmitate (El-Assaad et al., 2010; Han et al., 2010). Briefly, the cells were incubated in their respective medium with 25 mM glucose (high glucose) in the presence of 0.5% BSA alone as a control or 0.5 mM palmitate bound to 0.5% BSA as the glucolipotoxic condition. Glucolipotoxicity was induced in β-cells for 24 h. At the same time, cells were treated with PEHS extract and quercetin derivatives to determine the effect of these treatments on glucolipotoxicity-induced oxidative stress. On the other hand, 3T3-L1 cells were differentiated and then glucolipotoxicity was induced for 7 days. Finally, PEHS extract and quercetin derivatives were assayed for 48 h.

2.4. Determination of intracellular ROS by H_2DCF-DA analysis, evaluation of lipid content by AdipoRed™ and determination of secreted adipokines by ELISA

Intracellular ROS generation was measured in both 3T3-L1 adipocytes and INS 832/13 pancreatic β-cells using H_2DCF-DA. Briefly, 2x H_2DCF-DA was added when the cells were ready to be analyzed. After 30 min at 37 °C, the cells were carefully washed with PBS (pH 7.4), and intracellular ROS levels were measured using a fluorescence microplate reader (POLARstar, Omega, BMG LABTECH) at 485 nm excitation and 520 nm emission or a multimode reader (Cytation 3, Biotek, Spain) at 495 nm excitation and 529 nm emission. The cells were photographed by using a fluorescence microscope (Eclipse TE2000-U, Nikon microscope, Melville, NY) at 10x.

The lipid content of hypertrophic adipocytes was assessed using the AdipoRed™ reagent. Briefly, the supernatant was removed from the cells, and the cells were washed carefully with PBS. Then, AdipoRed™ was added, and the cells were incubated for 15 min at room temperature. Triglyceride accumulation was measured using the POLARstar microplate reader at 485 nm excitation and 572 nm emission. Fat droplets were photographed using a fluorescence microscope (Eclipse TE2000-U) at 10x.

The concentrations of adipokines were determined in the serum-free supernatant of hypertrophic adipocytes by a Mouse Obesity ELISA Strip Analysis kit (Signosis, Inc., Sunnyvale, CA, USA), which determines 8 cytokines (leptin, TNF-α, insulin-like growth factor-1 (IGF-1), IL-6, vascular endothelial growth factor (VEGF), IL-1α, IL-1β and CCL2), following the manufacturer's instructions.

2.5. Quantification of AMPK, pAMPK, PPARα and FASN by Western blot

Hypertrophic adipocytes treated with PEHS extract, Q and Q3GA for 48 h were washed with PBS and lysed with radioimmunoprecipitation (RIPA) buffer (BioRad Laboratories Inc., Madrid Spain) for 20 min at −80 °C. Then, the samples were centrifuged to remove the cell debris. The protein concentrations were quantified using the Pierce BCA Protein Assay Kit (Thermo Scientific, Waltham, MA, USA). AMPK, phospho-AMPK (pAMPK), fatty acid synthase (FASN), peroxisome proliferator-activated receptor alpha (PPARα) and β-actin were analyzed by Western blotting. Electrophoresis was performed with 10% SDS precast polyacrylamide gels (BioRad Laboratories Inc., Madrid, Spain). Proteins were transferred to nitrocellulose membranes using the transfer system Trans-Blot® Turbo™ (BioRad Laboratories Inc.). The antibodies used were mouse anti-AMPK (ab80039, Abcam, Cambridge, UK), rabbit anti-pAMPK (Thr172) (2535, Cell Signaling Tech.), rabbit anti-FASN (ab128870, Abcam), rabbit anti-PPARα (ab24509, Abcam) and rabbit anti-β-actin (MABT523, Merck Millipore, Darmstadt, Germany).The secondary antibodies were goat anti-rabbit-HRP (A0545, Sigma-Aldrich) or goat anti-mouse-HRP (A9044, Sigma-Aldrich). Chemiluminescent detection was performed using the SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Scientific), and membranes were analyzed in a ChemiDoc system (Bio-Rad, Spain). Protein levels were quantified by band densitometry normalized to the β-actin signal using Image Lab software (Version 4.1, Bio-Rad, Madrid, Spain).

2.6. Plasmids and analysis of the human CCL2 gene enhancer

The pGL3-basic plasmid was obtained from Promega (Madison, WI), and the pGLM-ENH plasmid was obtained from Osaka University (Osaka, Japan). The pGLM-ENH plasmid contains an enhancer region between nucleotides 22742 to 22513 from the human CCL2 gene (230 bp) and an enhancer region between nucleotides 2107 to 2160 from the same gene (167 bp) coupled to the luciferase gene (Ueda et al., 1997) and linked to the pGL3-basic plasmid.

Differentiated adipocytes were harvested by trypsinization (HyQtase, Thermo Scientific). Transfection was performed using 0.05 μg of pGLM-ENH plasmid or pGL3-basic (empty) plasmid as a control. To obtain high transfection efficiency, plasmids were introduced into differentiated adipocytes using the Neon® Transfection System from Invitrogen, following the manufacturer's instructions. Briefly, the adipocytes and corresponding plasmids were submitted to 2 pulses at 1400 V for 20 ms. Moreover, plasmid-free adipocytes were or were not submitted to the microporation process, and both conditions were used as controls (data not shown). Then, the cells were seeded in high glucose medium and incubated 2 days later with PEHS extract or quercetin derivatives for 48 h. Finally, the cells were washed with PBS and lysed, and luciferase activity was determined using the Luciferase Assay System (Promega). Light production was measured using a luminometer (POLARstar).

2.7. Molecular docking

The structures of Q (PubChem CID: 5280343) and Q3GA (PubChem CID: 5274585) were obtained from the National Center for Biotechnology Information (NCBI) PubChem database (http://www.ncbi.nlm.nih.gov/pccompound). Human PPARα (1.65 Å resolution, PDB code **4P54**), FASN (2.3 Å resolution, PDB code **5C37**) and AMPK (2.63 Å resolution, PDB code **5ISO**) structures were obtained from the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank (PDB). The specific structure of each protein was visualized using PyMol 2.0 software (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC, at http://www.pymol.org/) without further optimization. Molecular docking experiments were performed using the YASARA v18.12.27 software (Krieger et al., 2004; Krieger and Vriend, 2014) that executed the AutoDock 4 algorithm (Morris et al., 2008), in

which 999 flexible docking runs were set and clustered (7 Å) around the putative binding sites. In our calculations, two complexed compounds belonged to different clusters if the ligand root-mean-square deviation (RMSD) of their atomic positions was larger than 7 Å. The AMBER99 force field was used. The YASARA pH command was set to 7.4. The YASARA software calculated the Gibbs free energy variation (ΔG, kcal/mol) between the target (human PPARα, FASN and AMPK) and the ligand (Q and Q3GA) in each binding position, with highly positive energy values indicating strong binding. All the values shown in the table's results included a negative sign. Moreover, the ΔG value for only the best compound docked in each cluster is shown. Dissociation constants were recalculated from the average binding energy of all the compounds in each cluster. The number of docked molecules (Q or Q3GA) included in each cluster of compounds is indicated as percent "Members". The theoretical dissociation constant of each ligand (Q or Q3GA) at its putative binding site can be determined by calculating the binding energy of the ligand-receptor complex. All the figures were prepared using PyMol 2.0 software. The interaction maps of quercetins (Q or Q3GA) with the different protein structures have been created with the PLIP algorithm (Salentin et al., 2015).

2.8. Analysis of mitochondrial mass and transmission electron microscopy

The mitochondrial mass of treated hypertrophic adipocytes was measured by using the fluorescent probe MitoTracker™ Green FM (Molecular Probes, Invitrogen, Carlsbad, CA, USA). MitoTracker Green accumulates in the mitochondrial matrix, thereby allowing the probe abundance to indicate the amount of active mitochondria. In brief, the medium was removed, and the fluorescent dye was added at 200 nM in medium and incubated for 45 min at 37 °C. Then, the staining solution was replaced with PBS, and fluorescence was quantified at 490/520 nm using a cell imaging multimode microplate reader (Cytation 3). Representative microphotographs of stained mitochondrial mass were taken with a GFP filter cube at 20x.

Hypertrophic adipocytes were collected after trypsin digestion, washed with PBS and fixed with a 2% glutaraldehyde solution in 0.1 M cacodylate buffer at a pH of 7.4. Samples were then postfixed in 1% osmium tetroxide (OsO4) for 2 h and dehydrated in graded acetone solutions prior to impregnation in increasing concentrations of resin in acetone over a 24 h period. Semithin sections (500 nm) were stained with 1% toluidine blue. Ultrathin sections (70 nm) were subsequently cut using a diamond knife, double-stained with uranyl acetate and lead citrate, and examined with a transmission electron microscope (Hitachi, Tokyo, Japan).

2.9. Statistical analyses

The results are expressed as the mean ± standard deviation of the mean from at least three independent experiments. Differences between two or more groups were compared by nonparametric tests and were considered statistically significant at $p < 0.05$. The stimulating or inhibitory effects of extracts and pure compounds versus the control were analyzed by one-way ANOVA and Tukey's test for multiple comparisons. All statistical analyses and graphic representations were performed using GraphPad Prism 5 software (GraphPad, San Diego, CA). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ indicate significant differences versus the high glucose control, unless otherwise stated.

3. Results

3.1. Selection of polyphenols and metabolites derived from HS extract

Several metabolites derived from HS extracts have been found in pharmacokinetic studies performed in a rat model that may account for such effects, i.e., organic acids, phenolic acid derivatives and flavonol-conjugated forms (Herranz-Lopez et al., 2017b). Evidence from our laboratory suggests that flavonol-conjugated forms seem to be the most potent candidates that contribute to the health effects of PEHS extract (Herranz-Lopez et al., 2017b; Joven et al., 2012). These compounds are more potent antioxidants than phenolic acids such as chlorogenic acid, a reference antioxidant that is also abundant in the extract. Therefore, we selected chlorogenic acid, Q, Q3GA and PEHS extract to be compared in the development of the present study.

3.2. PEHS extract and quercetin derivatives show antioxidant effects under glucolipotoxic conditions in different cell types

To identify the main contributors of the antioxidant activity of PEHS extract, the antioxidant capacity of the extract and the above selected compounds (chlorogenic acid, Q and Q3GA) (Herranz-Lopez et al., 2017b) was determined with 3T3-L1 hypertrophic adipocytes under high glucose-induced glucolipotoxic conditions, using ascorbic acid (or vitamin C) as a reference antioxidant (Chambial et al., 2013).

The two forms of quercetin showed a higher capacity to inhibit glucolipotoxicity-associated oxidative stress than the extract and the other compounds analyzed at the different concentrations tested. ROS generation was reduced to 37% and 44% of the control with Q and Q3GA, respectively (Fig. 1A). Furthermore, among the compounds, Q3GA exerted the most powerful inhibition of ROS generation under these conditions, suggesting that this metabolite could be one of the candidates for the potent antioxidant capacity of PEHS extract *in vivo*. Nevertheless, ascorbic acid and chlorogenic acid barely inhibited ROS generation in this cell model. Fluorescence microscopy observations of cells treated with the quercetin derivatives revealed a significant reduction in intracellular ROS generation at the end of the assay (Fig. 1B).

Nevertheless, hyperglycemia and hyperlipidemia occur together with type 2 diabetes, aggravating the progression of the disease. In this context and as previously shown, exposure to high glucose combined with high palmitate concentrations has been shown to increase intracellular ROS generation in 3T3-L1 adipocytes (Han et al., 2010). Therefore, PEHS extract and quercetin derivatives were also tested on 3T3-L1 adipocytes for 48 h under this highly pathological glucolipotoxic condition. Compared with the control, the extract and the two compounds tested significantly decreased oxidative stress in 3T3-L1 adipocytes. In this particular case, Q was the most potent compound (30 μg/mL PEHS extract: 42% mean value reduction; 100 μM Q: 57% mean value reduction; 100 μM Q3GA: 41% mean value reduction) (Fig. 1C).

Glucolipotoxicity has been shown to increase ROS production in INS 832/13 pancreatic β-cells (El-Assaad et al., 2010), which exhibit β-cell functionality to produce and secrete insulin. When PEHS extract and quercetin derivatives were incubated with β-cells under glucolipotoxic conditions (incubation with high glucose + palmitate) for 24 h, both the extract and the pure compounds significantly inhibited intracellular ROS production at the highest concentrations tested (30 μg/mL PEHS extract: 41% mean value reduction; 100 μM Q: 65% mean value reduction; 100 μM Q3GA: 64% mean value reduction) (Fig. 1D).

3.3. PEHS extract and quercetin derivatives modulate proinflammatory adipokine secretion under glucolipotoxic conditions

Given the marked antioxidant effects of the two forms of quercetin on adipocytes and pancreatic β-cells, we compared the potential effects of these compounds and PEHS extract on proinflammatory cytokine secretion in high glucose-induced hypertrophic adipocytes. We focused on a selection of cytokines, i.e., leptin, IGF-1, IL-6, VEGF, IL-1α, IL-1β, TNF-α and CCL2, which are the highly relevant in the development of insulin resistance and in inflammation (Makki et al., 2013).

The results revealed that, compared with the control, PEHS extract significantly inhibited the secretion of all these proinflammatory cytokines (Fig. 2A and B). In addition, the two forms of quercetin showed

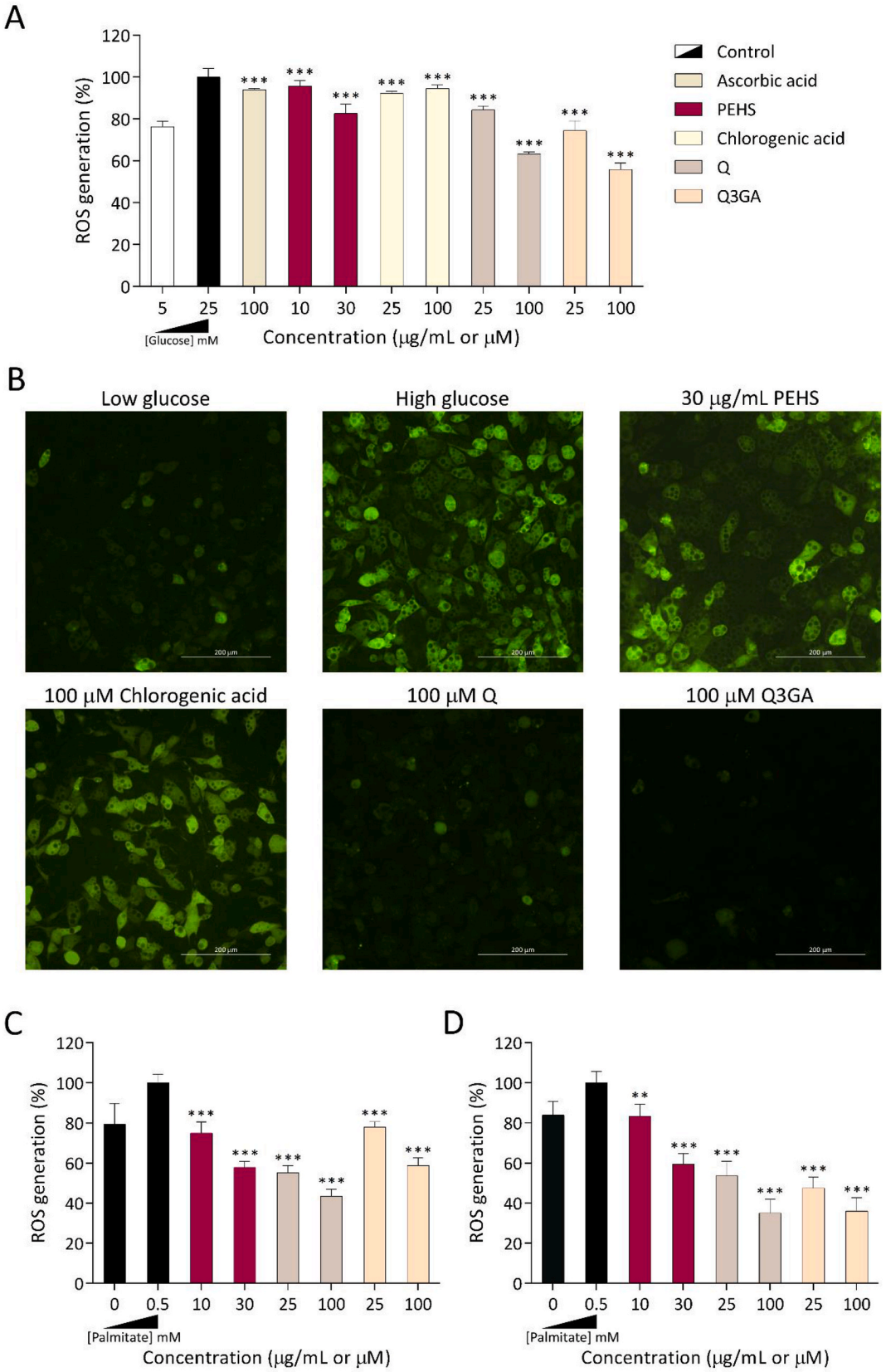

Fig. 1. PEHS extract and quercetin derivatives decrease ROS generation in hypertrophic 3T3-L1 adipocytes and INS 832/13 pancreatic β-cells. A) Measurement of intracellular ROS levels (%) using the H_2DCF-DA fluorescent probe in 3T3-L1 hypertrophic adipocytes following incubation with PEHS extract and different HS metabolites and antioxidants for 48 h under glucolipotoxic conditions. B) Representative fluorescence images of treated cells are shown. C) Measurement of intracellular ROS levels (%) in hypertrophic adipocytes (C) or pancreatic β-cells (D) under glucolipotoxic conditions after treatment with different concentrations of PEHS extract, Q or Q3GA for 48 h. Statistical analysis was performed as described in the Materials section.

comparable inhibitory capacities under the same conditions for most of the cytokines evaluated.

3.4. PEHS extract and quercetin derivatives modulate CCL2 expression at the transcriptional level

CCL2 is of particular interest (Fig. 2B) due to its capacity to attract macrophages to hypertrophic adipose tissue and to generate and amplify the inflammatory condition. To determine whether the downregulation of CCL2 by PEHS extract and quercetin derivatives occurred at the transcriptional level, the extract and compounds were incubated in adipocytes transfected with the enhancer region of the CCL2 promoter coupled to the luciferase gene (pGLM-ENH) (Ueda et al., 1997) and CCL2 gene expression was determined by luciferase activity (Fig. 2C).

As would be expected, adipocytes transfected with pGLM-ENH showed significantly increased luciferase activity compared to control adipocytes under high glucose-induced glucolipotoxic conditions. However, PEHS extract significantly reduced luciferase activity up to 36.6% compared to the control transfected with pGLM-ENH, indicating that the extract modulated CCL2 expression at the transcriptional level. Similarly, quercetin derivatives were also able to inhibit the expression of CCL2 to a similar extent (24.8% and 32.5% with Q and Q3GA, respectively) (Fig. 2C). Altogether, these findings offer a plausible explanation of the mechanism by which these compounds and the extract exert their action to reduce CCL2 levels.

3.5. PEHS extract and quercetin derivatives exhibit antilipogenic effects under glucolipotoxic conditions

Despite the previously observed antioxidant effects of ascorbic and chlorogenic acids, none of these compounds significantly modulated lipid content (Fig. 3A). Furthermore, no effect was observed for PEHS extract and Q at the lowest concentrations tested. Nevertheless, both PEHS extract and the two forms of quercetin (Q and Q3GA) at the highest concentration tested exhibited significant differences from the high glucose control (Fig. 3A), reducing the accumulated triglyceride content by 20%, 15% and 27%, respectively. Q3GA reduced triglyceride content even at 25 μM, demonstrating a marked antilipogenic capacity after incubation under high glucose concentrations. Microscopic observations of treated cells confirmed that cytoplasmic triglyceride accumulation was significantly decreased with the PEHS extract and quercetin derivative treatments compared to the control condition (Fig. 3B).

3.6. PEHS extract and quercetin derivatives modulate the activity and expression of proteins related to energy and lipid metabolism

To understand the underlying mechanism by which quercetin derivatives from HS reduce triglyceride accumulation, we selected several key molecular targets related to lipid homeostasis and energy metabolism in adipose tissue: AMPK, FASN and PPARα. AMPK plays an important role in cellular energy metabolism and is activated by

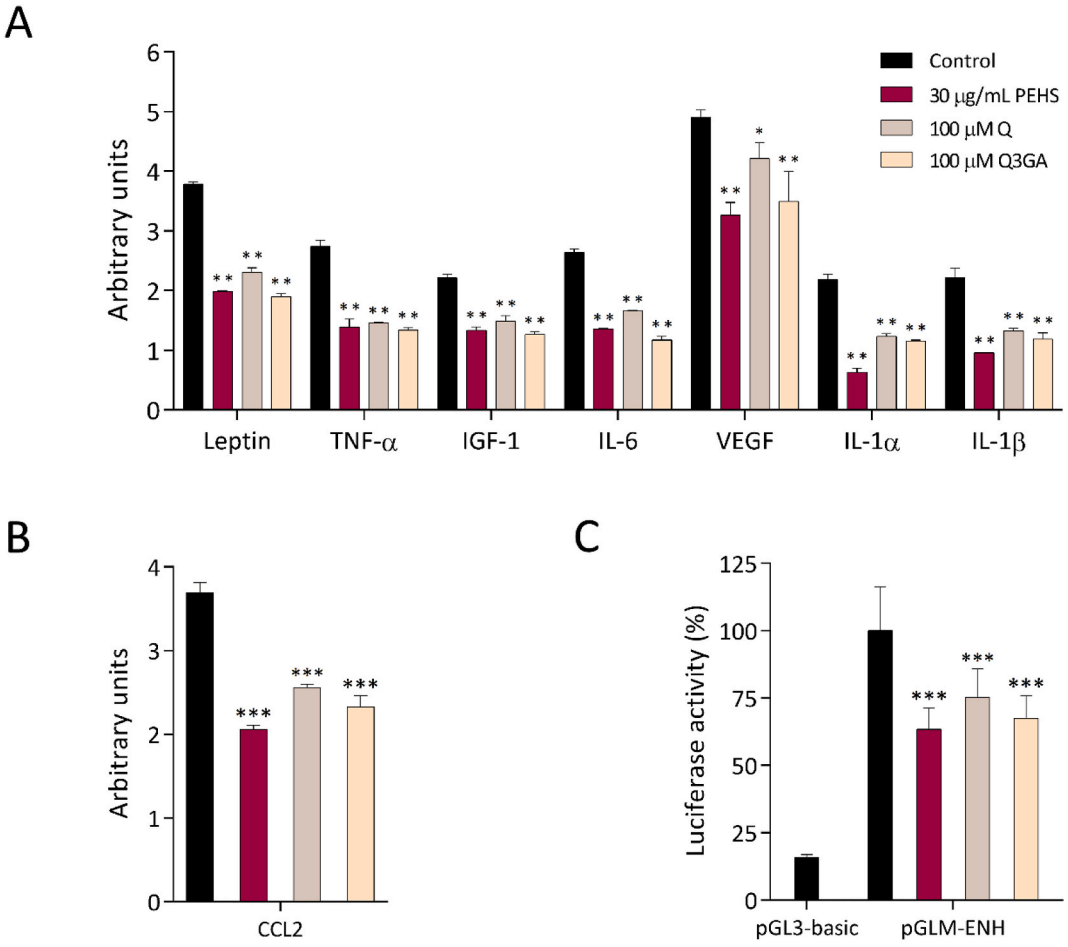

Fig. 2. PEHS extract and quercetin derivatives modulate adipokine secretion under glucolipotoxic conditions. A and B) Hypertrophic adipocytes were treated for 48 h with PEHS extract and quercetin derivatives at the most active concentrations tested, and adipokine levels were determined by ELISA. C) 3T3-L1 adipocytes transfected with the enhancer region of the CCL2 promoter were treated with PEHS extract, Q or Q3GA at different concentrations for 48 h, and CCL2 gene expression was determined by luciferase activity. Statistical analysis was performed as described in the Materials section.

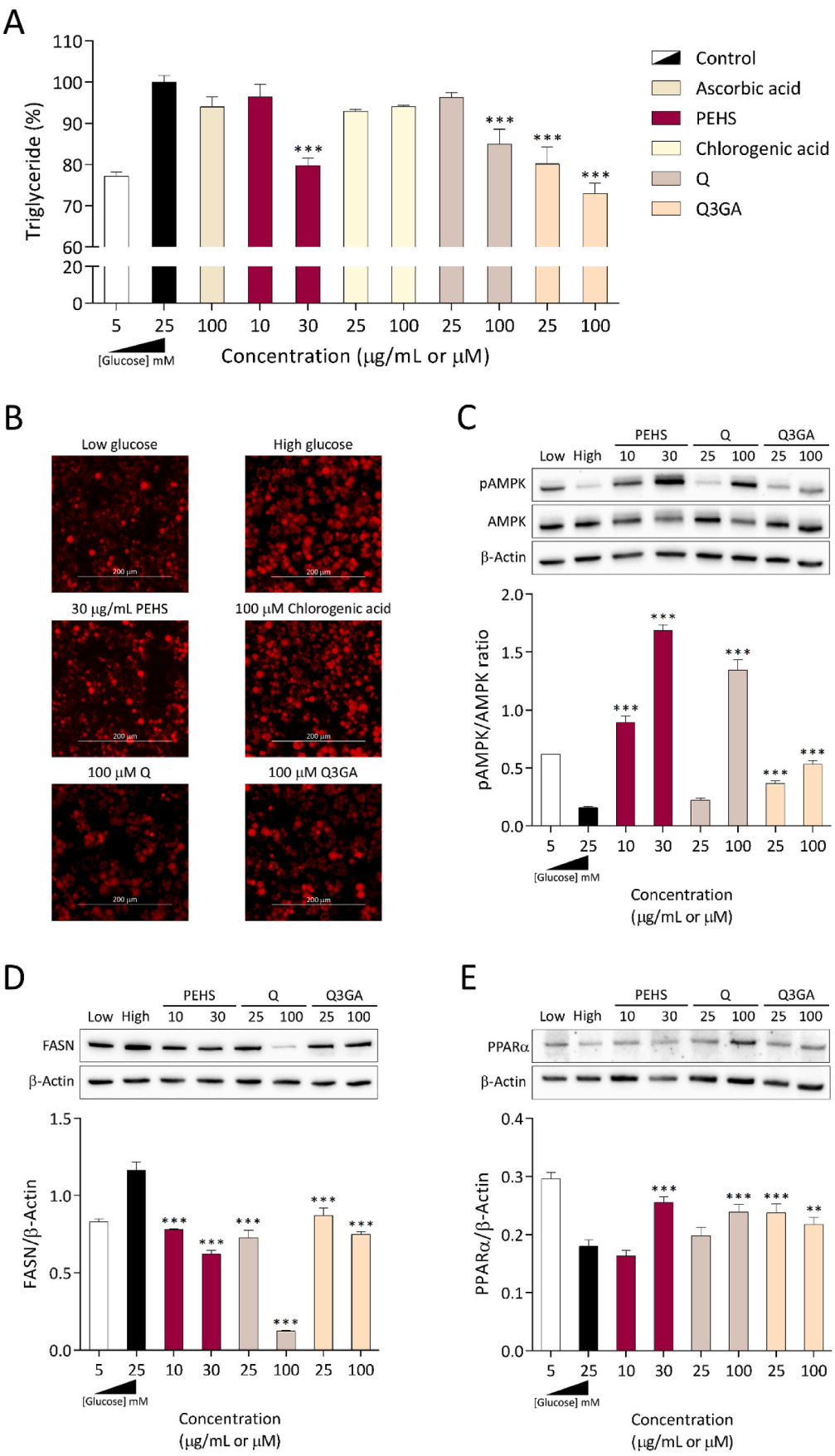

Fig. 3. PEHS extract and quercetin derivatives exhibit antilipogenic effects under glucolipotoxic conditions by modulating the activity and expression of proteins related to energy and lipid metabolism. A and B) High glucose-induced hypertrophic 3T3-L1 adipocytes were incubated with PEHS extract or pure compounds for 48 h, and the accumulated triglyceride content was determined by AdipoRed™ staining. The effect was confirmed by microscopic observations of treated cells stained with AdipoRed™. C, D and E) Measurement of AMPK activation and expression levels of FASN and PPARα by Western blot analysis in hypertrophic adipocytes treated with PEHS extract, Q and Q3GA for 48 h. Statistical analysis was performed as described in the Materials section.

phosphorylation (pAMPK). FASN is the last enzyme of the lipogenic pathway. PPARα is the main regulator of lipid metabolism, and its activation induces lipid catabolism through genes related to fatty acid mobilization, transport and oxidation.

PEHS extract, Q and Q3GA were assayed for 48 h in hypertrophic adipocytes, and the extract and both compounds significantly activated AMPK by phosphorylation (activation is expressed as the pAMPK/AMPK ratio), with the polyphenolic extract being more active than the pure compounds (Fig. 3C). In addition, PEHS extract, Q and Q3GA significantly reduced FASN expression (Fig. 3D) and increased PPARα expression (Fig. 3E). Nevertheless, in the first case, the metabolite Q was more active than PEHS extract and Q3GA at modulating FASN expression.

3.7. *In silico analysis of quercetin derivatives reveals a potential therapeutic drug for obesity treatment*

To explore the possibility that quercetin derivatives acted as direct modulators of the previously studied protein targets, a global molecular docking procedure was performed. The obtained results are shown in Fig. 4, Supplementary Tables 1 to 3, and Supplementary Figs. 1–3. A first observation of the obtained results showed that for the three proteins of interest, the clusters with low ΔG for both compounds were located in functionally relevant positions previously described in the literature. For both Q and Q3GA, the best clusters of each compound were located in the broad binding cavity described for physiological agonists of PPARα. However, the best positions for Q3GA showed higher theoretical affinity (low ΔG, approximately −11.44 kcal/mol, see Fig. 4B and Supplementary Table 1) than those for Q (approximately −9.5 kcal/mol, see Fig. 4A and Supplementary Table 1). Both Q and Q3GA were predicted to

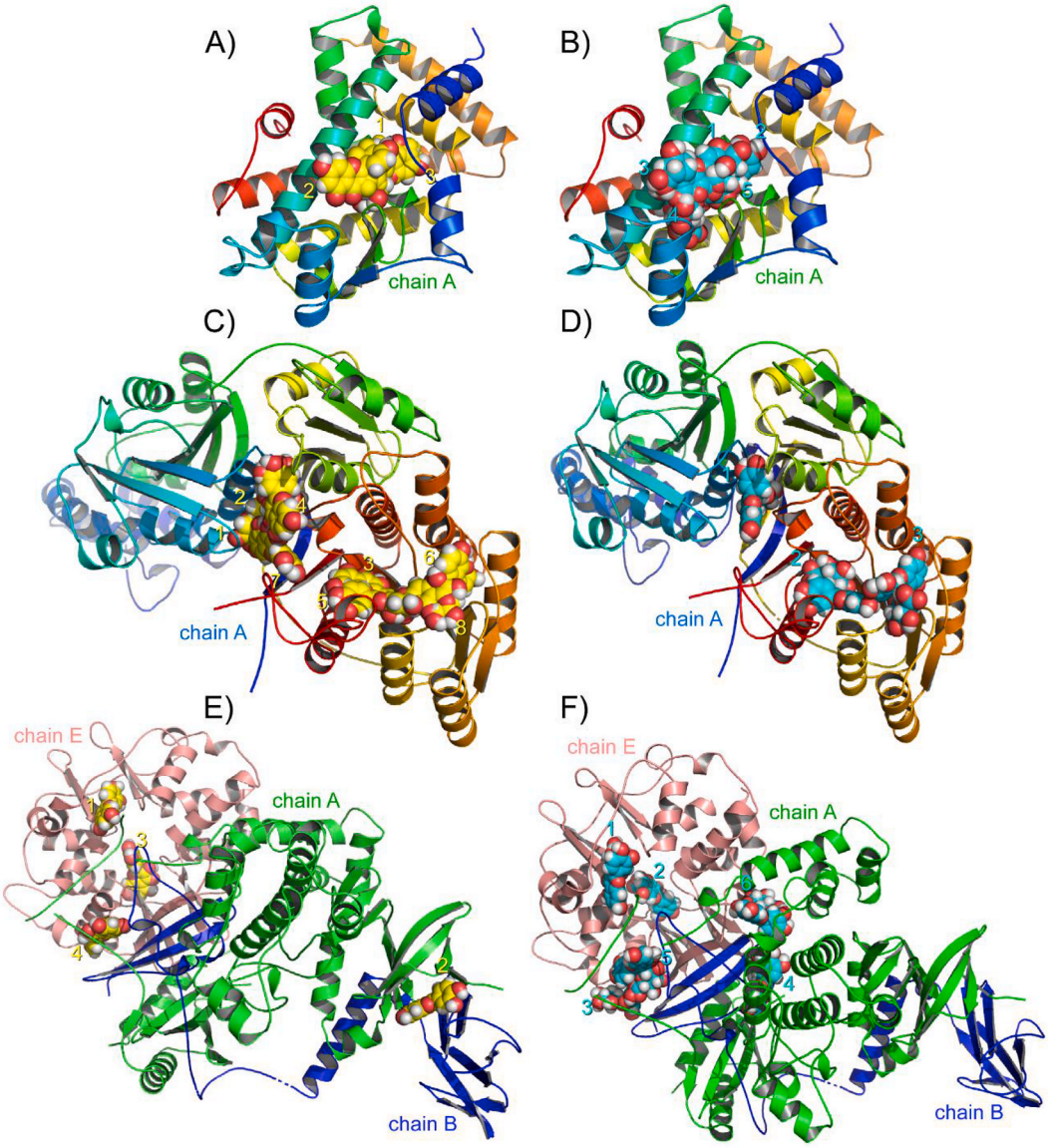

Fig. 4. Illustration of the secondary structure of human PPARα (A, B), FASN (C, D), and AMPK (E, F) with docked Q (A, C, E) and Q3GA (B, D, F). For each cluster of the docked compound (Q carbon atoms are shown in yellow, and Q3GA carbon atoms are shown in cyan), the molecules (spheres) with high binding energy are shown. For PPARα (A, B) and FASN (C, D), the secondary structure is shown in rainbow colors from N-terminal (blue) to C-terminal (red). The three AMPK subunits are shown with different colors (alpha-2 is shown in green as *chain A*, beta-1 is shown in blue as *chain B*, and gamma-1 is shown in salmon as *chain E*). The number of each cluster is indicated. The figure was prepared using PyMol 2.0 software. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

possess a good interaction with PPARα. The interaction is stabilized by 9 hydrogen bonds for the best Q cluster (Tyr214, Tyr214, Lys22, Thr283, Glu286, Ser323, Tyr334, Tyr334, Asp372). All residues present at the binding site. In addition, various hydrophobic interactions Phe281, Thr279, Met320, Val324 (See Supplementary Fig. 1 and Table 1) also stabilized the complex. Similarly, the predicted interaction for Q3GA occupies locations very close to those of Q in the PPARα ligand-binding cavity. The stabilization of Q3GA-PPARα complex is due to both hydrogen bonds (Thr279, Thr283, Thr283, His440, Tyr464) and hydrophobic interactions (Phe273, Thr279, Tyr314, Ile317, Leu321), Supplementary Fig. 1. A similar analysis showed that the best clusters for both compounds docked on the deep cavity of the FASN catalytic domain, which under physiological conditions should be occupied by NADPH and a nascent fatty acid. The calculated affinity for Q3GA (ΔG approximately −13.67 kcal/mol, see Fig. 4D, and Supplementary Table 2 and Supplementary Fig. 2) was higher than that for Q (ΔG -11.28 kcal/mol, and Supplementary Table 2 and Supplementary Fig. 2), and in that location these compounds would behave as competitive inhibitors capable of reducing fatty acid biosynthesis. Both Q-FASN- and Q3GA-FASN-complex are stabilized mostly by hydrogen bonds, up to 14 (Asp214, Glu218, Glu218, Asn438, Asn438, Arg441, Arg441, Arg442, Phe494, Arg495, His496, His496, Arg652, Arg652) for Q3GA-FASN-complex, and up to 11 (Glu115, Lys210, Leu244, His248, Phe494, Arg495, Arg495, His496, His496, Arg652, Arg652) in Q-FASN-complex. There are also hydrophobic interactions, although quantitatively minor (see Supplementary Fig. 2). Finally, molecular docking simulations for both Q and Q3GA showed that the clusters with the highest calculated affinity were located in the AMPKγ regulatory subunit (see Fig. 4E and F, and Supplementary Table 3 and Supplementary Fig. 3). Specifically, they occupied previously described sites that bind AMP (see chain E in Fig. 4E and F). The detail of the protein-quercetin interaction maps can be seen in Supplementary Fig. 3. It should be noted that among the interactions that stabilize the Q3GA-AMPK complexes, saline bridges are present for several clusters (Arg269/E, Arg299/E for cluster 1; Arg224/E, Lys243/E for cluster 2; Lys260/A for cluster 4; Lys47/E, Lys399/A for cluster 5; and Lys259/B, Lys260/B, Arg331/A for cluster 6), in addition to hydrogen bonds and hydrophobic interactions. Both compounds would behave, therefore, as AMPK agonists. It should be noted that cluster 2 for Q (see Fig. 4E) was located in the binding site in the established zone of interaction between the AMPKα catalytic and AMPKβ regulatory subunits. This site has been described as a binding site for the AMPK activator called A-769662 (Langendorf et al., 2016). It is expected that the binding of Q at the same position also activated the enzyme. In addition, neither of the two compounds significantly bound to the catalytic site of the AMPKα subunit.

3.8. PEHS extract and the metabolite Q3GA improve mitochondrial biogenesis and restore mitochondrial dynamics

In adipocytes, severe defects in mitochondrial biogenesis and altered mitochondrial dynamics induce triglyceride accumulation and have been linked to defects in the secretion of anti-inflammatory adipokines (Vankoningsloo et al., 2005). Considering the inhibitory effect of PEHS extract and quercetin derivatives on the secretion of proinflammatory factors and their antilipogenic capacity, we evaluated the potential action of these compounds on mitochondrial remodeling. For this purpose, the differential effects of PEHS extract, Q and Q3GA on mitochondrial mass were evaluated by using a mitochondrial-selective fluorescent probe. Adipocytes under glucolipotoxic conditions develop mitochondrial stress, showing a significant decrease in mitochondrial content (Gao et al., 2010). In high glucose-induced hypertrophic cells, our results show an upward trend (not significant) in mitochondrial mass and biogenesis upon Q incubation relative to the control. Further, the administration of PEHS extract and Q3GA increased mitochondrial mass under glucolipotoxic conditions (Fig. 5A). Fluorescence photomicrographs showed a significant increase in the mitochondrial content of PEHS extract and Q3GA-treated cells compared to that of high glucose-induced hypertrophic adipocytes (Fig. 5B).

Furthermore, to determine whether PEHS extract and Q3GA had any effect on mitochondrial dynamics, we assessed the ultrastructure of mitochondria in adipocytes after treatment with PEHS extract and Q3GA. Transmission electron microscopy observations showed imbalanced mitochondrial dynamics in high glucose-induced hypertrophic adipocytes (Fig. 5C). Fusion and fission, processes that determine mitochondrial turnover, were overrepresented in these cells. This altered mitochondrial dynamics was also accompanied by the appearance of autophagosome-related structures (Fig. 5C). This situation could occur as part of a protective mechanism against glucolipotoxic conditions, in an effort to preserve mitochondrial bioenergetics capacities and eliminate nonviable mitochondria through autophagy. This compromised mitochondrial viability seemed to be recovered by PEHS extract and Q3GA, since neither fusion-fission processes nor autophagic vacuole structures were observed in adipocytes treated with these agents under glucolipotoxic conditions (Fig. 5C).

4. Discussion

It has been postulated that dietary polyphenols could be used as therapeutic agents for the treatment of obesity (Joven et al., 2014). Different cell lines have been widely used as a model to study metabolic disturbances related to obesity. Despite the limitations of a cell model, the 3T3-L1 adipocytic cell line under glucolipotoxic conditions is a well-established cell model to study the effect of these phytochemicals and their metabolites on obesity-associated metabolic disturbances. Moreover, pancreatic β-cells under similar conditions represents a valuable resource for spotting potential candidate regulators of insulin resistance and obesity (El-Assaad et al., 2010; Gao et al., 2010; Vankoningsloo et al., 2005). In these models, *in vitro* incubation with high glucose and palmitic acid mimics the physiological condition of an excess of nutrients, induces cell hypertrophy and simulates a state of inflammation and insulin resistance, similar to that observed under obese conditions (Han et al., 2010; Tanis et al., 2015). In addition, the excessive intracellular lipid storage that occurs in adipocytes as a result of exposure to high extracellular glucose concentrations is another way to mimic glucolipotoxic conditions; this treatment leads to metabolic alterations, mitochondrial dysfunction and increased oxidative stress (Mittra et al., 2008).

Considering that complex mixtures of polyphenols are able to interact with different metabolic targets(Barrajon-Catalan et al., 2014), reliable *in vitro* tools are required to identify the particular compounds in the mixture responsible for the biological activity and to dissect their putative mechanism. Thus, the identification of these candidates will allow us to establish additional plausible approaches and develop synergistic mixtures with improved success rates in clinical trials focused on metabolic disturbances associated with obesity.

Previous evidence has demonstrated that HS might represent a relevant therapeutic tool in the treatment and prevention of obesity and related diseases (Herranz-Lopez et al., 2017b; Hopkins et al., 2013). HS extract contains several compounds such as anthocyanins, organic acids, phenolic acids and flavonoids (Borrás-Linares et al., 2015). Nevertheless, recent data provide evidence that flavonols, especially quercetin derivatives, are the main compounds responsible for the salutary effects of the extract (Fernandez-Arroyo et al., 2012; Joven et al., 2012). Q and its metabolite Q3GA were reported to be the flavonol derivatives with the highest concentrations (C_{max} values of 1.57 and 3 μM, respectively) in the plasma of rats after the acute ingestion of PEHS extract (Fernandez-Arroyo et al., 2012), suggesting that these compounds might be the main candidates for the bioactivity of the whole extract. Indeed, there was a correlation between the long half-life elimination values of quercetin glucuronides and the lipid peroxidation inhibition in plasma after the oral administration of the PEHS extract. In agreement with our results, other authors have reported that quercetin glucuronides are the

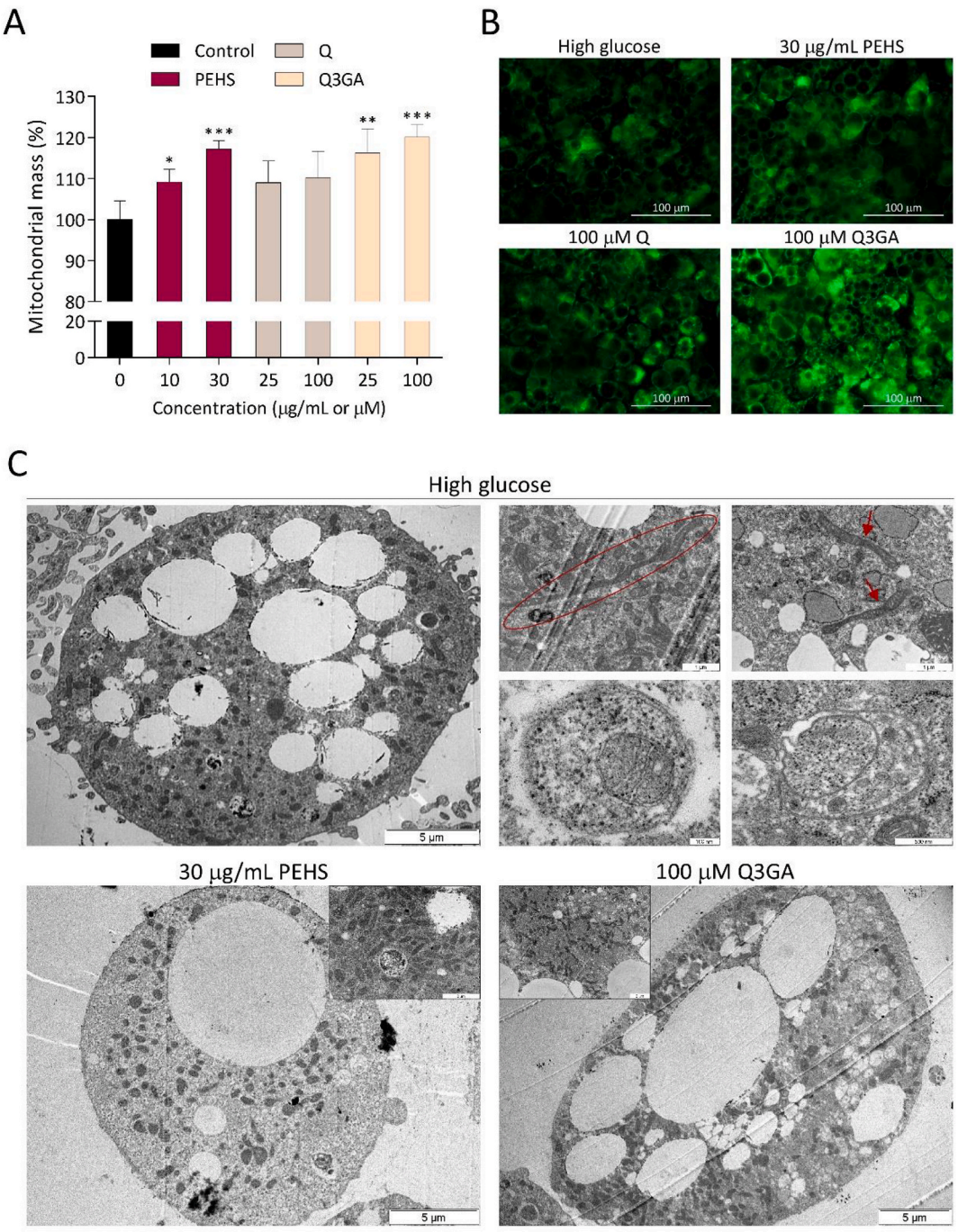

Fig. 5. PEHS extract and quercetin derivatives restore mitochondrial mass and integrity. A) High glucose-induced hypertrophic 3T3-L1 adipocytes were incubated with PEHS extract, Q or Q3GA for 48 h, and mitochondrial mass was determined by the fluorescent probe MitoTracker™ Green FM. B) Representative fluorescence images of treated cells are shown. C) Transmission electron microscopy images representative of the mitochondria in 3T3-L1 hypertrophic adipocytes treated with PEHS extract at 30 μg/mL or Q and Q3GA at 100 μM. Circles show fused mitochondria, and arrows show fission events. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

most representative quercetin-conjugated forms after the consumption of quercetin-enriched plant sources in both humans and rats (Sesink et al., 2001; Yang et al., 2016). Consistent with these results, Q3GA was detected using an anti-Q3GA antibody in the liver tissue of hyperlipidemic mice fed PEHS extract (Joven et al., 2012). Because of these findings, we aimed to study the capacity of selected metabolites derived from HS extract to alleviate glucolipotoxicity-induced metabolic stress and to reach target tissues, and we compared these metabolites with the whole extract in hypertrophic 3T3-L1 adipocytes and pancreatic β-cells under glucolipotoxic conditions. We chose the most common quercetin metabolites found in plasma (Q and Q3GA) and a metabolite derived from phenolic acids, i.e., chlorogenic acid (the ester of caffeic acid and (−)-quinic acid).

Our results showed that both Q and Q3GA seemed to be more effective than chlorogenic acid, ascorbic acid and PEHS extract in reducing ROS generation in adipocytes under glucolipotoxic conditions. These results were in agreement with the strong antioxidant capacity observed for Q in a large number of *in vitro* and animal models (Kobori et al., 2011; Li et al., 2016; Makris and Rossiter, 2001; Nabavi et al., 2012; Roche et al., 2009; Sanchez-Reus et al., 2007; Vidyashankar et al.,

2013), which indicate that this flavonol and its derivatives may directly act as a free radical scavenger or act through the activation of antioxidant enzymes. The weak capacity of chlorogenic acid to reduce intracellular ROS generation might be related to the difficult metabolism of this compound as an intracellular metabolite in the cell model. In a previous study, it was estimated that the concentration of total flavonols present in adipocytes treated with 30 μg/mL of PEHS extract was approximately 4 μM of total flavonols (Herranz-Lopez et al., 2012). Therefore, it is reasonable to expect that the whole extract had a lower capacity to decrease intracellular ROS than Q or Q3GA. The decreased capacity observed for the whole extract compared to the quercetin derivatives could be explained by the aforementioned aspect and by the limited conversion of the glycosylated flavonols in the extract into flavonol-conjugated metabolites that may reach their intracellular targets. The concentrations of flavonol-conjugated metabolites utilized in these cell models may be higher than those found in plasma samples (within the low micromolar range). However, the required dose of HS extract in humans to be continually used in nutritional interventions focused on obesity management should be verified in further clinical studies.

Regarding the molecular mechanism involved, recent evidence suggests that high levels of glucose induce hypertrophy and CCL2 expression and secretion in adipocytes, which leads to macrophage recruitment and chronic inflammation (Han et al., 2007; Joven et al., 2013). However, not only high levels of glucose but also the combination of high glucose with saturated fatty acids such as palmitate induces inflammation in adipose tissue (Han et al., 2010), a mechanism that seems to be mediated by ROS generation. Therefore, we also analyzed the capacity of PEHS extract and quercetin derivatives to inhibit oxidative stress in 3T3-L1 adipocytes and INS 832/13 pancreatic β-cells under glucolipotoxic conditions. Our results showed that both the extract and the two quercetin derivatives maintained their antioxidant properties in both cell models. The capacity to reduce oxidative stress in INS 832/13 pancreatic β-cells may mean a positive effect on β-cell viability that could be instrumental in type 2 diabetes management.

Both PEHS extract and quercetin derivatives were able to reduce the levels of proinflammatory cytokines in an adipocyte model (leptin, TNF-α, IGF-1, IL-6, VEGF, IL-1α, IL-1β and CCL2). The anti-inflammatory activity of Q has been widely demonstrated in many cells and animal studies (Dong et al., 2014; Indra et al., 2013; Overman et al., 2011; Vidyashankar et al., 2013; Ying et al., 2013). This activity seems to be exerted through the direct modulation of the gene expression of proinflammatory proteins, likely by epigenetic control, or through the modulation of the main transcription factor NFκB (Indra et al., 2013; Overman et al., 2011; Panchal et al., 2012; Ying et al., 2013). CCL2 was found at high levels in the adipose tissue of obese people (Han et al., 2007; Kim and Park, 2015), and it is postulated that the regulation of the metabolic pathways responsible for the production of this cytokine might prevent chronic inflammatory diseases, such as those associated with obesity. Therefore, CCL2 or its receptor are postulated to be therapeutic targets to treat the inflammation associated with obesity. The results obtained from the transfection experiments confirmed that both PEHS extract and quercetin derivatives were able to reduce CCL2 gene expression. Again, the anti-inflammatory effect of the whole extract was almost comparable to the quercetin derivatives, indicating that compounds in the extract other than flavonols may be involved in this effect.

The two forms of quercetin and PEHS extract also significantly reduced triglyceride accumulation in hypertrophic adipocytes. In contrast, chlorogenic acid and ascorbic acid did not affect triglyceride content. This result is in agreement with previous results obtained in cell lines and animal models (Ahn et al., 2008; Dong et al., 2014; Imessaoudene et al., 2016; Jung et al., 2013; Kobori et al., 2011; Vidyashankar et al., 2013; Ying et al., 2013), in which Q showed antiadipogenic and antilipogenic properties and was able to modify tissue lipid composition and reduce fat deposition and weight gain *in vivo*. These data suggest that Q and its glucuronidated derivative may be applied to the treatment of obesity and its related diseases due to antioxidant properties or lipid metabolism regulation. Considering the strong capacity of Q and Q3GA to decrease triglyceride accumulation in the hypertrophic adipocyte model, we aimed to elucidate the underlying potential mechanism, focusing on several protein targets closely related to energy metabolism and lipid metabolism regulation. Therefore, we determined the comparative capacity of PEHS extract and both quercetin derivatives to modulate AMPK, FASN and PPARα in 3T3-L1 adipocytes under glucolipotoxic conditions. The results from Western blot analysis suggested that Q and its glucuronidated metabolite have a significant role in decreasing the accumulation of triglycerides via the modulation of these three metabolic targets.

AMPK is a key sensor on energy metabolism (Hwang et al., 2009). AMPK is a serine/threonine kinase that is activated by phosphorylation when the AMP:ATP ratio increases, activating catabolic pathways and inhibiting anabolic pathways. AMPK is a crucial regulator of the balance between energy supply and energy demand on cells. Thus, AMPK phosphorylates metabolic enzymes such as acetyl-CoA carboxylase (ACC), a central enzyme in lipid biosynthesis. When phosphorylated by AMPK, ACC is inhibited and reduces the levels of malonyl-CoA, an allosteric inhibitor of carnitine palmitoyl transferase 1 (CPT1) (Saha and Ruderman, 2003). The subsequent activation of CPT1 promotes fatty acid transport to the mitochondrial matrix where β-oxidation occurs. Western blot analysis revealed that PEHS extract, Q and Q3GA activated AMPK via phosphorylation in a dose-dependent manner. The fact that the extract showed a stronger effect than the quercetin metabolites suggests again that other compounds in the extract may also be implicated. Therefore, AMPK activation could be partly responsible for the decrease in lipid content that could be induced by the reduction in lipogenesis via inhibiting ACC and/or by increasing fatty acid oxidation. Recently, the capacity of Q to activate AMPK has been demonstrated in obese C57BL/6J mice (Dong et al., 2014), which suggests that the activation of this kinase is involved in the suppression of inflammation and insulin resistance in mouse adipose tissue. Therefore, our results provide a reasonable basis to postulate that some plasma compounds and metabolites from PEHS extract, probably quercetin derivatives, decrease fat deposition and inflammation via AMPK activation when ingested by mice.

Similarly, Western blot analysis revealed that PEHS extract, Q3GA and particularly Q were able to reduce FASN expression levels in adipocytes under glucolipotoxic conditions. This protein is a crucial enzyme in the *de novo* synthesis of saturated long-chain fatty acids. The antilipogenic effect of PEHS extract, Q and Q3GA suggests a complementary action to the inhibition of ACC by AMPK that results in lipolysis, and both effects can explain the observed global decrease in adipocyte triglycerides (Menendez et al., 2009). The inhibition of FASN expression by AMPK activation has been previously reported to be induced either by the AMPK activator AICAR or by the anti-diabetic drug metformin in liver and cancer cells (Xiang et al., 2004; Zhou et al., 2001), most likely due to the strong suppressive effect of AMPK on sterol regulatory element-binding protein-1c (SREBP1c) expression. Whether the effect of PEHS extract or its quercetin metabolites on FASN expression and activation is a consequence of the capacity to activate AMPK or whether these compounds are able to directly modulate FASN may deserve further research.

Therefore, we postulate that Q and Q3GA may be one of the main compounds in PEHS extract responsible for decreasing lipid accumulation in hypertrophic adipocytes via a reduction in FASN expression and the activation of mitochondrial fatty acid oxidation. Furthermore, these data are in agreement with the results obtained in hyperlipidemic mice fed PEHS extract. In this animal model, the presence of Q3GA in the liver tissue of these mice is correlated with the activation of AMPK and the decreased expression of lipogenic genes such as SREBP1c and FASN (Joven et al., 2012).

Considering that the concentration of total flavonols in the 10 μg/mL and 30 μg/mL PEHS extract treatments was approx. 1.3 and 4 μM,

respectively, the increased capacity of PEHS extract to decrease the intracellular ROS in both cell models treated with high concentrations of palmitic acid compared to that of the pure compounds was remarkable. Moreover, PEHS extract also showed a potent ability to decrease triglyceride content, to activate AMPK and to decrease FASN expression in hypertrophic adipocytes. These results may indicate that compounds other than flavonols likely contribute to the capacity of PEHS extract to alleviate glucolipotoxicity-induced metabolic stress.

Our results also demonstrated that PEHS extract, Q and Q3GA were able to increase PPARα levels. This transcription factor is a main modulator of the expression of genes related to peroxisomal and mitochondrial fatty acid oxidation, mitochondrial biogenesis and antioxidant enzyme synthesis, and its activation results in the fatty acid catabolism, reduced lipid tissue content, and decreased glucolipotoxicity and oxidative stress (Okada-Iwabu et al., 2013). Therefore, we postulate that the reduction in fat accumulation mediated by PEHS extract, Q and Q3GA was partially due to their antilipogenic effect and also due to an increase in fatty acid oxidation via PPARα activation, preserving mitochondrial function and content. Our results also support that PEHS extract and Q3GA revert the decrease in mitochondrial mass induced by glucolipotoxicity in hypertrophic adipocytes and restore mitochondrial biogenesis and viability. Proper oxidative mitochondrial function may contribute to the observed decreased lipid content induced by these agents. Considering that mitochondrial dysfunction is partially associated with a reduction in mitochondrial content in obese conditions (Jokinen et al., 2017), Q3GA could prevent mitochondrial degradation. In line with our results, it was reported that Q and apigenin ameliorate the metabolic stress-induced alterations in mitochondria by decreasing ROS, improving membrane potential and mitochondrial biogenesis in 3T3-L1 adipocytes (Nisha et al., 2014). These results are also consistent with the activation of PPARα observed in obese C57BL/6J mice fed Q (Kobori et al., 2011), which significantly reduced liver triglycerides and oxidative stress. In agreement to our results, isorhamnetin, a methylated derivative of Q, promoted mitochondrial biogenesis in 3T3-L1 adipocytes through mitochondrial gene expression, mitochondrial DNA replication and AMPK activation (Lee and Kim, 2018).

In correlation with these molecular findings, our *in silico* studies have also demonstrated the potential of quercetin derivatives to interact with several protein targets as part of their putative mechanism. In our study, Q3GA showed a lower ΔG than Q for PPARα and FASN, which indicated that Q3GA is a promising candidate to act as a direct modulator of both proteins, as described for other polyphenols (Encinar et al., 2015; Olivares-Vicente et al., 2018). This fact underwrites the capacity of this compound to modulate lipid metabolism and mitochondrial function beyond simple antioxidant capacity. In addition, molecular docking experiments showed that both quercetin derivatives seemed to be capable of acting as AMPK agonists and interacting with the AMPKγ regulatory subunit. Nevertheless, the strong activation of AMPK induced by PEHS extract may indicate that other compounds may be involved in the activation of this enzyme.

We have previously demonstrated that both Q and Q3GA were absorbed by hypertrophic adipocytes and intracellularly metabolized to some extent to Q3GA and Q, respectively (Herranz-Lopez et al., 2017a). Although the absorption of Q was more efficient and faster than that of Q3GA, both compounds were intracellularly localized and inhibited ROS generation in a similar manner. Our results confirm that Q and Q3GA show similar antioxidant and anti-inflammatory capacities in adipocyte models under glucolipotoxic conditions. Furthermore, both compounds present a high capacity to reduce triglyceride accumulation, an effect that occurred through FASN downregulation, AMPK activation and mitochondrial mass and viability restoration via PPARα upregulation. All of these data suggest that Q and Q3GA could be the main candidates responsible for the bioactive effects of whole PEHS extract that revert glucolipotoxicity-induced metabolic stress in adipose tissue. Interestingly, the strong antilipogenic and AMPK-activating capacity observed for the whole extract may deserve further research to elucidate the other compounds that contribute to this capacity.

CRediT authorship contribution statement

María Herranz-López: Conceptualization, Formal analysis, Investigation, Project administration, Supervision, Writing - original draft, Writing - review & editing. **Mariló Olivares-Vicente:** Conceptualization, Formal analysis, Investigation, Methodology, Writing - original draft, Writing - review & editing. **Esther Rodríguez Gallego:** Data curation, Formal analysis, Writing - original draft. **Jose Antonio Encinar:** Data curation, Formal analysis, Methodology, Writing - original draft, Writing - review & editing. **Almudena Pérez-Sánchez:** Data curation. **Verónica Ruiz-Torres:** Data curation. **Jorge Joven:** Funding acquisition. **Enrique Roche:** Funding acquisition, Project administration. **Vicente Micol:** Conceptualization, Funding acquisition, Investigation, Project administration, Supervision, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2020.111606.

References

Addabbo, F., Montagnani, M., Goligorsky, M.S., 2009. Mitochondria and reactive oxygen species. Hypertension. Dallas, Tex. 53, 885–892, 1979.

Ahn, J., Lee, H., Kim, S., Park, J., Ha, T., 2008. The anti-obesity effect of quercetin is mediated by the AMPK and MAPK signaling pathways. Biochem. Biophys. Res. Commun. 373, 545–549.

Alarcon-Aguilar, F.J., Zamilpa, A., Perez-Garcia, M.D., Almanza-Perez, J.C., Romero-Nunez, E., Campos-Sepulveda, E.A., Vazquez-Carrillo, L.I., Roman-Ramos, R., 2007. Effect of *Hibiscus sabdariffa* on obesity in MSG mice. J. Ethnopharmacol. 114, 66–71.

Barrajon-Catalan, E., Herranz-Lopez, M., Joven, J., Segura-Carretero, A., Alonso-Villaverde, C., Menendez, J.A., Micol, V., 2014. Molecular promiscuity of plant polyphenols in the management of age-related diseases: far beyond their antioxidant properties. Adv. Exp. Med. Biol. 824, 141–159.

Beltran-Debon, R., Alonso-Villaverde, C., Aragones, G., Rodriguez-Medina, I., Rull, A., Micol, V., Segura-Carretero, A., Fernandez-Gutierrez, A., Camps, J., Joven, J., 2010. The aqueous extract of Hibiscus sabdariffa calices modulates the production of monocyte chemoattractant protein-1 in humans. Phytomedicine 17, 186–191.

Bhatti, J.S., Bhatti, G.K., Reddy, P.H., 2017. Mitochondrial dysfunction and oxidative stress in metabolic disorders — a step towards mitochondria based therapeutic strategies. Biochim. Biophys. Acta 1863, 1066–1077.

Borrás-Linares, I., Herranz-López, M., Barrajón-Catalán, E., Arráez-Román, D., González-Álvarez, I., Bermejo, M., Fernández Gutiérrez, A., Micol, V., Segura-Carretero, A., 2015. Permeability study of polyphenols derived from a phenolic-enriched Hibiscus sabdariffa extract by UHPLC-ESI-UHR-qq-TOF-MS. Int. J. Mol. Sci. 16, 18396–18411.

Cancello, R., Clement, K., 2006. Is obesity an inflammatory illness? Role of low-grade inflammation and macrophage infiltration in human white adipose tissue. Bjog 113, 1141–1147.

Chambial, S., Dwivedi, S., Shukla, K.K., John, P.J., Sharma, P., 2013. Vitamin C in disease prevention and cure: an overview. Indian J. Clin. Biochem. 28, 314–328.

Chang, H.C., Peng, C.H., Yeh, D.M., Kao, E.S., Wang, C.J., 2014. *Hibiscus sabdariffa* extract inhibits obesity and fat accumulation, and improves liver steatosis in humans. Food Funct 5, 734–739.
Despres, J.P., Lemieux, I., 2006. Abdominal obesity and metabolic syndrome. Nature 444, 881–887.
Dong, J., Zhang, X., Zhang, L., Bian, H.X., Xu, N., Bao, B., Liu, J., 2014. Quercetin reduces obesity-associated ATM infiltration and inflammation in mice: a mechanism including AMPKalpha1/SIRT1. J. Lipid Res. 55, 363–374.
El-Assaad, W., Joly, E., Barbeau, A., Sladek, R., Buteau, J., Maestre, I., Pepin, E., Zhao, S., Iglesias, J., Roche, E., Prentki, M., 2010. Glucolipotoxicity alters lipid partitioning and causes mitochondrial dysfunction, cholesterol, and ceramide deposition and reactive oxygen species production in INS832/13 ss-cells. Endocrinology 151, 3061–3073.
Encinar, J.A., Fernandez-Ballester, G., Galiano-Ibarra, V., Micol, V., 2015. In silico approach for the discovery of new PPARgamma modulators among plant-derived polyphenols. Drug Des. Dev. Ther. 9, 5877–5895.
Fernandez-Arroyo, S., Herranz-Lopez, M., Beltran-Debon, R., Borras-Linares, I., Barrajon-Catalan, E., Joven, J., Fernandez-Gutierrez, A., Segura-Carretero, A., Micol, V., 2012. Bioavailability study of a polyphenol-enriched extract from Hibiscus sabdariffa in rats and associated antioxidant status. Mol. Nutr. Food Res. 56, 1590–1595.
Fernandez-Arroyo, S., Rodriguez-Medina, I., Beltran-Debon, R., Pasini, F., Joven, J., Micol, V., Segura-Carretero, A., Fernandez-Gutierrez, A., 2011. Quantification of the polyphenolic fraction and *in vitro* antioxidant and *in vivo* anti-hyperlipemic activities of *Hibiscus sabdariffa* aqueous extract. Food Res. Int. 44.
Gao, C.L., Zhu, C., Zhao, Y.P., Chen, X.H., Ji, C.B., Zhang, C.M., Zhu, J.G., Xia, Z.K., Tong, M.L., Guo, X.R., 2010. Mitochondrial dysfunction is induced by high levels of glucose and free fatty acids in 3T3-L1 adipocytes. Mol. Cell. Endocrinol. 320, 25–33.
Green, H., Kehinde, O., 1975. An established preadipose cell line and its differentiation in culture. II. Factors affecting the adipose conversion. Cell 5, 19–27.
Han, C.Y., 2016. Roles of reactive oxygen species on insulin resistance in adipose tissue. Diabetes Metab. J 40, 272–279.
Han, C.Y., Kargi, A.Y., Omer, M., Chan, C.K., Wabitsch, M., O'Brien, K.D., Wight, T.N., Chait, A., 2010. Differential effect of saturated and unsaturated free fatty acids on the generation of monocyte adhesion and chemotactic factors by adipocytes: dissociation of adipocyte hypertrophy from inflammation. Diabetes 59, 386–396.
Han, C.Y., Subramanian, S., Chan, C.K., Omer, M., Chiba, T., Wight, T.N., Chait, A., 2007. Adipocyte-derived serum amyloid A3 and hyaluronan play a role in monocyte recruitment and adhesion. Diabetes 56, 2260–2273.
Herranz-Lopez, M., Borras-Linares, I., Olivares-Vicente, M., Galvez, J., Segura-Carretero, A., Micol, V., 2017a. Correlation between the cellular metabolism of quercetin and its glucuronide metabolite and oxidative stress in hypertrophied 3T3-L1 adipocytes. Phytomedicine 25, 25–28.
Herranz-Lopez, M., Fernandez-Arroyo, S., Perez-Sanchez, A., Barrajon-Catalan, E., Beltran-Debon, R., Menendez, J.A., Alonso-Villaverde, C., Segura-Carretero, A., Joven, J., Micol, V., 2012. Synergism of plant-derived polyphenols in adipogenesis: perspectives and implications. Phytomedicine 19, 253–261.
Herranz-Lopez, M., Olivares-Vicente, M., Encinar, J.A., Barrajon-Catalan, E., Segura-Carretero, A., Joven, J., Micol, V., 2017b. Multi-targeted molecular effects of Hibiscus sabdariffa polyphenols: an opportunity for a global approach to obesity. Nutrients 9.
Hopkins, A.L., Lamm, M.G., Funk, J.L., Ritenbaugh, C., 2013. *Hibiscus sabdariffa* L. in the treatment of hypertension and hyperlipidemia: a comprehensive review of animal and human studies. Fitoterapia 85, 84–94.
Hwang, J.T., Kwon, D.Y., Yoon, S.H., 2009. AMP-activated protein kinase: a potential target for the diseases prevention by natural occurring polyphenols. New biotechnology 26, 17–22.
Imessaoudene, A., Merzouk, H., Berroukeche, F., Mokhtari, N., Bensenane, B., Cherrak, S., Merzouk, S.A., Elhabiri, M., 2016. Beneficial effects of quercetin-iron complexes on serum and tissue lipids and redox status in obese rats. J. Nutr. Biochem. 29, 107–115.
Indra, M.R., Karyono, S., Ratnawati, R., Malik, S.G., 2013. Quercetin suppresses inflammation by reducing ERK1/2 phosphorylation and NF kappa B activation in leptin-induced human umbilical vein endothelial cells (HUVECs). BMC Res. Notes 6, 275.
Jokinen, R., Pirnes-Karhu, S., Pietiläinen, K.H., Pirinen, E., 2017. Adipose tissue NAD+-homeostasis, sirtuins and poly(ADP-ribose) polymerases -important players in mitochondrial metabolism and metabolic health. Redox Biology 12, 246–263.
Joven, J., Espinel, E., Rull, A., Aragones, G., Rodriguez-Gallego, E., Camps, J., Micol, V., Herranz-Lopez, M., Menendez, J.A., Borras, I., Segura-Carretero, A., Alonso-Villaverde, C., Beltran-Debon, R., 2012. Plant-derived polyphenols regulate expression of miRNA paralogs miR-103/107 and miR-122 and prevent diet-induced fatty liver disease in hyperlipidemic mice. Biochim. Biophys. Acta 1820, 894–899.
Joven, J., Micol, V., Segura-Carretero, A., Alonso-Villaverde, C., Menéndez, J.A., Aragonès, G., Barrajón-Catalán, E., Beltrán-Debón, R., Camps, J., Cufí, S., Fernández-Arroyo, S., Fernández-Gutiérrez, A., Guillén, E., Herranz-López, M., Iswaldi, I., Lozano-Sánchez, J., Martin-Castillo, B., Oliveras-Ferraros, C., Pérez-Sánchez, A., Rodríguez-Gallego, E., Rull, A., Saura, D., Vázquez-Martín, A., 2014. Polyphenols and the modulation of gene expression pathways: can we eat our way out of the danger of chronic disease? Crit. Rev. Food Sci. Nutr. 54, 985–1001.
Joven, J., Rull, A., Rodriguez-Gallego, E., Camps, J., Riera-Borrull, M., Hernandez-Aguilera, A., Martin-Paredero, V., Segura-Carretero, A., Micol, V., Alonso-Villaverde, C., Menendez, J.A., 2013. Multifunctional targets of dietary polyphenols in disease: a case for the chemokine network and energy metabolism. Food Chem. Toxicol. 51, 267–279.
Jung, C.H., Cho, I., Ahn, J., Jeon, T.I., Ha, T.Y., 2013. Quercetin reduces high-fat diet-induced fat accumulation in the liver by regulating lipid metabolism genes. Phytother Res. 27, 139–143.
Kim, J.K., So, H., Youn, M.J., Kim, H.J., Kim, Y., Park, C., Kim, S.J., Ha, Y.A., Chai, K.Y., Kim, S.M., Kim, K.Y., Park, R., 2007. *Hibiscus sabdariffa* L. water extract inhibits the adipocyte differentiation through the PI3-K and MAPK pathway. J. Ethnopharmacol. 114, 260–267.
Kim, T.K., Park, K.S., 2015. Inhibitory effects of harpagoside on TNF-alpha-induced pro-inflammatory adipokine expression through PPAR-gamma activation in 3T3-L1 adipocytes. Cytokine 76, 368–374.
Kobori, M., Masumoto, S., Akimoto, Y., Oike, H., 2011. Chronic dietary intake of quercetin alleviates hepatic fat accumulation associated with consumption of a Western-style diet in C57/BL6J mice. Mol. Nutr. Food Res. 55, 530–540.
Krieger, E., Darden, T., Nabuurs, S.B., Finkelstein, A., Vriend, G., 2004. Making optimal use of empirical energy functions: force-field parameterization in crystal space. Proteins 57, 678–683.
Krieger, E., Vriend, G., 2014. YASARA View - molecular graphics for all devices - from smartphones to workstations. Bioinformatics 30, 2981–2982.
Langendorf, C.G., Ngoei, K.R.W., Scott, J.W., Ling, N.X.Y., Issa, S.M.A., Gorman, M.A., Parker, M.W., Sakamoto, K., Oakhill, J.S., Kemp, B.E., 2016. Structural basis of allosteric and synergistic activation of AMPK by furan-2-phosphonic derivative C2 binding. Nat. Commun. 7, 10912.
Lee, M.S., Kim, Y., 2018. Effects of isorhamnetin on adipocyte mitochondrial biogenesis and AMPK activation. Molecules 23.
Li, C., Zhang, W.J., Frei, B., 2016. Quercetin inhibits LPS-induced adhesion molecule expression and oxidant production in human aortic endothelial cells by p38-mediated Nrf2 activation and antioxidant enzyme induction. Redox Biol 9, 104–113.
Luna-Luna, M., Medina-Urrutia, A., Vargas-Alarcon, G., Coss-Rovirosa, F., Vargas-Barron, J., Perez-Mendez, O., 2015. Adipose tissue in metabolic syndrome: onset and progression of atherosclerosis. Arch. Med. Res. 46, 392–407.
Maestre, I., JordáN, J., Calvo, S., Reig, J.A., Ceña, V., Soria, B., Prentki, M., Roche, E., 2003. Mitochondrial dysfunction is involved in apoptosis induced by serum withdrawal and fatty acids in the β-cell line INS-1. Endocrinology 144, 335–345.
Makki, K., Froguel, P., Wolowczuk, I., 2013. Adipose tissue in obesity-related inflammation and insulin resistance: cells, cytokines, and chemokines. ISRN inflammation 2013, 139239–139239.
Makris, D.P., Rossiter, J.T., 2001. Comparison of quercetin and a non-orthohydroxy flavonol as antioxidants by competing *in vitro* oxidation reactions. J. Agric. Food Chem. 49, 3370–3377.
Menendez, J.A., Vazquez-Martin, A., Ortega, F.J., Fernandez-Real, J.M., 2009. Fatty acid synthase: association with insulin resistance, type 2 diabetes, and cancer. Clin. Chem. 55, 425–438.
Mittra, S., Bansal, V.S., Bhatnagar, P.K., 2008. From a glucocentric to a lipocentric approach towards metabolic syndrome. Drug Discov. Today 13, 211–218.
Miyake, H., Sakagami, J., Yasuda, H., Sogame, Y., Kato, R., Suwa, K., Dainaka, K., Takata, T., Yokota, I., Itoh, Y., 2018. Association of fatty pancreas with pancreatic endocrine and exocrine function. PLoS One 13, e0209448.
Morris, G.M., Huey, R., Olson, A.J., 2008. Using AutoDock for Ligand-Receptor Docking. Current Protocols in Bioinformatics Chapter 8, Unit 8.14.
Nabavi, S.M., Nabavi, S.F., Eslami, S., Moghaddam, A.H., 2012. *In vivo* protective effects of quercetin against sodium fluoride-induced oxidative stress in the hepatic tissue. Food Chem. 132, 931–935.
Nemeth, K., Piskula, M.K., 2007. Food content, processing, absorption and metabolism of onion flavonoids. Crit. Rev. Food Sci. Nutr. 47, 397–409.
Nisha, V.M., Anusree, S.S., Priyanka, A., Raghu, K.G., 2014. Apigenin and quercetin ameliorate mitochondrial alterations by tunicamycin-induced ER stress in 3T3-L1 adipocytes. Appl. Biochem. Biotechnol. 174, 1365–1375.
O'Neill, S., O'Driscoll, L., 2015. Metabolic syndrome: a closer look at the growing epidemic and its associated pathologies. Obes. Rev. 16, 1–12.
Okada-Iwabu, M., Yamauchi, T., Iwabu, M., Honma, T., Hamagami, K., Matsuda, K., Yamaguchi, M., Tanabe, H., Kimura-Someya, T., Shirouzu, M., Ogata, H., Tokuyama, K., Ueki, K., Nagano, T., Tanaka, A., Yokoyama, S., Kadowaki, T., 2013. A small-molecule AdipoR agonist for type 2 diabetes and short life in obesity. Nature 503, 493–499.
Olivares-Vicente, M., Barrajon-Catalan, E., Herranz-Lopez, M., Segura-Carretero, A., Joven, J., Encinar, J.A., Micol, V., 2018. Plant-derived polyphenols in human health: biological activity, metabolites and putative molecular targets. Curr. Drug Metabol. 19, 351–369.
Overman, A., Chuang, C.C., McIntosh, M., 2011. Quercetin attenuates inflammation in human macrophages and adipocytes exposed to macrophage-conditioned media. Int. J. Obes. 35, 1165–1172.
Panchal, S.K., Poudyal, H., Brown, L., 2012. Quercetin ameliorates cardiovascular, hepatic, and metabolic changes in diet-induced metabolic syndrome in rats. J. Nutr. 142, 1026–1032.
Roche, M., Tarnus, E., Rondeau, P., Bourdon, E., 2009. Effects of nutritional antioxidants on AAPH- or AGEs-induced oxidative stress in human SW872 liposarcoma cells. Cell Biol. Toxicol. 25, 635–644.
Rodriguez-Medina, I.C., Beltran-Debon, R., Molina, V.M., Alonso-Villaverde, C., Joven, J., Menendez, J.A., Segura-Carretero, A., Fernandez-Gutierrez, A., 2009. Direct characterization of aqueous extract of Hibiscus sabdariffa using HPLC with diode array detection coupled to ESI and ion trap MS. J. Separ. Sci. 32, 3441–3448.
Saha, A.K., Ruderman, N.B., 2003. Malonyl-CoA and AMP-activated protein kinase: an expanding partnership. Mol. Cell. Biochem. 253, 65–70.
Salentin, S., Schreiber, S., Haupt, V.J., Adasme, M.F., Schroeder, M., 2015. PLIP: fully automated protein–ligand interaction profiler. Nucleic Acids Res. 43, W443–W447.

Sanchez-Reus, M.I., Gomez del Rio, M.A., Iglesias, I., Elorza, M., Slowing, K., Benedi, J., 2007. Standardized *Hypericum perforatum* reduces oxidative stress and increases gene expression of antioxidant enzymes on rotenone-exposed rats. Neuropharmacology 52, 606–616.

Sesink, A.L., O'Leary, K.A., Hollman, P.C., 2001. Quercetin glucuronides but not glucosides are present in human plasma after consumption of quercetin-3-glucoside or quercetin-4'-glucoside J Nutr 131, 1938–1941.

Tanis, R.M., Piroli, G.G., Day, S.D., Frizzell, N., 2015. The effect of glucose concentration and sodium phenylbutyrate treatment on mitochondrial bioenergetics and ER stress in 3T3-L1 adipocytes. Biochim. Biophys. Acta Mol. Cell Res. 1853, 213–221.

Ueda, A., Ishigatsubo, Y., Okubo, T., Yoshimura, T., 1997. Transcriptional regulation of the human monocyte chemoattractant protein-1 gene. Cooperation of two NF-kappaB sites and NF-kappaB/Rel subunit specificity. J. Biol. Chem. 272, 31092–31099.

Vankoningsloo, S., Piens, M., Lecocq, C., Gilson, A., De Pauw, A., Renard, P., Demazy, C., Houbion, A., Raes, M., Arnould, T., 2005. Mitochondrial dysfunction induces triglyceride accumulation in 3T3-L1 cells: role of fatty acid beta-oxidation and glucose. J. Lipid Res. 46, 1133–1149.

Vidyashankar, S., Sandeep Varma, R., Patki, P.S., 2013. Quercetin ameliorate insulin resistance and up-regulates cellular antioxidants during oleic acid induced hepatic steatosis in HepG2 cells. Toxicol. Vitro 27, 945–953.

Xiang, X., Saha, A.K., Wen, R., Ruderman, N.B., Luo, Z., 2004. AMP-activated protein kinase activators can inhibit the growth of prostate cancer cells by multiple mechanisms. Biochem. Biophys. Res. Commun. 321, 161–167.

Yang, L.-L., Xiao, N., Li, X.-W., Fan, Y., Alolga, R.N., Sun, X.-Y., Wang, S.-L., Li, P., Qi, L.-W., 2016. Pharmacokinetic comparison between quercetin and quercetin 3-O-β-glucuronide in rats by UHPLC-MS/MS. Sci. Rep. 6, 35460.

Ying, H.Z., Liu, Y.H., Yu, B., Wang, Z.Y., Zang, J.N., Yu, C.H., 2013. Dietary quercetin ameliorates nonalcoholic steatohepatitis induced by a high-fat diet in gerbils. Food Chem. Toxicol. 52, 53–60.

Zhou, G., Myers, R., Li, Y., Chen, Y., Shen, X., Fenyk-Melody, J., Wu, M., Ventre, J., Doebber, T., Fujii, N., Musi, N., Hirshman, M.F., Goodyear, L.J., Moller, D.E., 2001. Role of AMP-activated protein kinase in mechanism of metformin action. J. Clin. Invest. 108, 1167–1174.