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Research Paper

The deleterious effect of cholesterol and protection by quercetin on mitochondrial bioenergetics of pancreatic β-cells, glycemic control and inflammation: *In vitro* and *in vivo* studies

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ABSTRACT

Studying rats fed high cholesterol diet and a pancreatic β-cell line (Min6), we aimed to determine the mechanisms by which quercetin protects against cholesterol-induced pancreatic β-cell dysfunction and impairments in glycemic control. Quercetin prevented the increase in total plasma cholesterol, but only partially prevented the high cholesterol diet-induced alterations in lipid profile. Quercetin prevented cholesterol-induced decreases in pancreatic ATP levels and mitochondrial bioenergetic dysfunction in Min6 cells, including decreases in mitochondrial membrane potentials and coupling efficiency in the mitochondrial respiration (basal and maximal oxygen consumption rate (OCR), ATP-linked OCR and reserve capacity). Quercetin protected against cholesterol-induced apoptosis of Min6 cells by inhibiting caspase-3 and -9 activation and cytochrome c release. Quercetin prevented the cholesterol-induced decrease in antioxidant defence enzymes from pancreas (cytosolic and mitochondrial homogenates) and Min6 cells and the cholesterol-induced increase of cellular and mitochondrial oxidative status and lipid peroxidation. Quercetin counteracted the cholesterol-induced activation of the NFκB pathway in the pancreas and Min6 cells, normalizing the expression of pro-inflammatory cytokines. Quercetin inhibited the cholesterol-induced decrease in *sirtuin 1* expression in the pancreas and pancreatic β-cells. Taken together, the anti-apoptotic, antioxidant and anti-inflammatory properties of quercetin, and its ability to protect and improve mitochondrial bioenergetic function are likely to contribute to its protective action against cholesterol-induced pancreatic β-cell dysfunction, thereby preserving glucose-stimulated insulin secretion (GSIS) and glycemic control. Specifically, the improvement of ATP-linked OCR and the reserve capacity are important mechanisms for protection of quercetin. In addition, the inhibition of the NFκB pathway is an important mechanism for the protection of quercetin against cytokine mediated cholesterol-induced glycemic control impairment. In summary, our data highlight cellular, molecular and bioenergetic mechanisms underlying quercetin's protective effects on β-cells *in vitro* and *in vivo*, and provide a scientifically tested foundation upon which quercetin can be developed as a nutraceutical to preserve β-cell function.

1. Introduction

Cholesterol plays an important role in pancreatic β-cell dysfunction. Mice with specific inactivation of ABCA1 (ATP-binding cassette transporter subfamily A member 1), a transporter that mediates reverse cholesterol efflux show impaired glucose tolerance and insulin secretion [1,2]. Moreover, a direct link has been found between elevated cholesterol and reduced insulin secretion in islets isolated from C57BL/6J mice and in INS-1 rat pancreatic β-cells [3], as well as in Min6 cells [4], whereby insulin secretion can be normalized through cholesterol depletion [3]. LDL receptor deficient mice exhibit hypercholesterolemia with elevated cholesterol levels in pancreatic islets, which is associated with β-cell

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dysfunction, impaired glucose tolerance and reduced glucose-stimulated insulin secretion (GSIS) [5]. Given that pancreatic β-cells are considered to be particularly susceptible to oxidative stress due to their relatively low antioxidant enzyme content [6], it has been suggested that cholesterol may induce β-cell dysfunction by promoting apoptosis through oxidative stress pathways [4,7] and mitochondrial damage [4,8] and also by altering membrane fluidity [9]. Although the molecular mechanism underlying cholesterol-induced inflammatory damage to β-cells is not well understood, it has been shown that cholesterol is able to increase TNF-α (tumor necrosis factor alpha), interleukin-6 (IL-6) and macrophage colony-stimulating factor (*m*-CSF) in macrophages [10], which could contribute to the damage of β-cells.

Quercetin (QUE) is a natural polyphenolic flavonoid which is believed to have widespread health benefits due to a combination of its properties. Quercetin can be (a) antioxidant, by free radical scavenging and induction of antioxidant defence via nuclear factor (erythroid-derived 2)-like 2 (Nrf2) activation, (b) anti-inflammatory through reducing pro- and increasing anti-inflammatory cytokines *via* nuclear factor kappa B (NFκB) inactivation and (c) anti-apoptotic by modulating JNK (c-Jun N-terminal kinase) and ERK (extracellular-signal-regulated kinase)-pathways [11–14]. Recent *in vitro* evidence showed the mitochondrial protective effect of QUE, restoring mitochondrial membrane potential (MMP), ATP levels and complex-I activity altered by indomethacin, a non-steroidal anti-inflammatory drug, in intestinal Caco-2 cells [15]. Interestingly, compared to other polyphenols like resveratrol, rutin and epigallocatechin gallate, QUE was the most efficient in protecting against mitochondrial dysfunction [15]; this could be due to its ability to enter cells and accumulate in mitochondria [15,16]. In addition, it has been shown that QUE up-regulates mitochondrial complex-I activity to protect against programmed cell death in rotenone model of Parkinson's disease in rats [17]. Quercetin also has been shown to increase markers of mitochondrial biogenesis, such as expression of *sirtuin 1* (*Sirt1*), a nicotinamide adenosine dinucleotide-dependent histone deacetylase, and peroxisome proliferator-activated receptor gamma coactivator-1-alpha (*PGC-1α*), in soleus muscle and brain; these changes were associated with an improvement in exercise performance [18].

Cytokine-induced pancreatic β-cell apoptosis is considered the main mechanism for β-cell death [19]. Cytokine-induced mitochondrial dysfunction through activation of the NFκB-pathway and oxidative stress may also trigger β-cell death [20–23]. Quercetin has been shown to prevent cytokine-induced pancreatic β-cell death by counteracting the mitochondrial apoptosis pathway and NFκB signalling, thereby preserving glucose-stimulated insulin secretion (GSIS) [20–23]. In addition, QUE *via* the ERK1/2 pathway, protects β-cells against oxidative damage [24]. Overexpression of *Sirt1* in β-cells improves GSIS, while *Sirt1* knockdown results in impaired response to glucose [25]. Given that impaired GSIS is a hallmark of the transition from the pre-diabetic to diabetic state [26], QUE has been proposed to be a promising anti-diabetic agent due to its ability to induce antioxidant effects through Sirt1. Also, QUE is known to protect β-cells against damage and to ameliorate hyperglycemia in diabetic animals by reducing oxidative stress, preserving β-cell mass, and lowering plasma glucose and cholesterol levels [27,28]. Based on evidence that QUE accumulates in mitochondria [15,16], its β-cell protective effects may rely not only in its anti-inflammatory and anti-oxidant properties, but also on its protection of mitochondrial function. The present study aimed to determine the mechanism underlying the protective effect of QUE on the impairment of GSIS in a pancreatic β-cell line exposed to cholesterol and glycemic control in rats fed a high-cholesterol diet. This study addresses the protective effects of QUE on mitochondrial bioenergetic dysfunction, inflammation, oxidative stress and apoptosis induced by high levels of cholesterol.

2. Materials and methods

2.1. Animals and diets

The study protocol was approved by the Animal Ethics Committee of the Faculty of Medicine of the University of Chile (Approval No. CBA# 0586 FMUCH) and all procedures were performed in compliance with the Guidelines for Care and Use of Laboratory Animals at the Faculty of Medicine. Male Wistar rats (90–110 g, 5–6 weeks old) from the Faculty of Medicine were housed in a 12 h light/dark schedule at room temperature with water *ad libitum*. Forty animals were randomly distributed into 5 groups and fed standard diet (AIN-76A/Clinton-Cybulsky Cholesterol Series #1-107); standard diet supplemented with quercetin (0.5% w/w); high-cholesterol (HC) diet (1.25% cholesterol w/w, AIN-76A/Clinton-Cybulsky Cholesterol Series #3-107); or HC diet supplemented with either quercetin (0.5% w/w) or ezetimibe (0.001% w/w) for 4 weeks (N=8 rats per group). Ezetimibe blocks Niemann-Pick C1 Like 1 (NPC1L1) protein in the small intestine, a transporter that mediates cholesterol absorption [29].

2.2. Laboratory determinations and sample collection

Tail blood glucose levels were determined after 12 h-fasted (from 9 pm) animals after 2 and 4 weeks of treatment using an Accu-chek glucometer (Roche, Mannheim, Germany). Immediately afterwards in 4 week treated rats a glucose tolerance test (IPGTT, 2 g/kg.i.p.) was assayed and blood glucose levels were determined from tail bleeds. Twenty four h after the IPGTT, plasma glucose and insulin levels were measured using a colorimetric kit and a rat insulin ELISA kit, respectively. Cholesterol levels were determined using a colorimetric colestat enzimática AA kit and pro-inflammatory cytokines using a magnetic bead Multiplex assay on plasma obtained from the inferior vena cava under ketamine:xylazine anaesthesia (100 mg/kg:10 mg/kg, i.p.). Lipid profile (LDL, VLDL and HDL) was performed by Laboratorio Clinico Medicina Nuclear (Santiago, Chile). Pancreases were removed and stored in 4% paraformaldehyde and embedded in paraffin for immunohistochemistry. Pancreatic tissue from the same rats was also stored in RNA later (Thermo Scientific, MA USA) for gene expression assays and at −80 °C for biochemical analyses, including the quantification of cholesterol using the Amplex® Red Cholesterol Assay Kit (Thermo Scientific, MA USA).

2.3. Cell culture

Min6 cells (p38–p51) were cultured in DMEM (25 mM glucose) supplemented with 10% heat-inactivated FBS, 100 IU/ml penicillin and 100 μg/ml streptomycin. All experiments were conducted in unsupplemented DMEM media. A “water-soluble cholesterol” containing 47 mg of cholesterol/g solid (molar ratio, 1:6 cholesterol/ methyl-β-cyclodextrin, Sigma, MO, USA) was used to deliver cholesterol to the cells [4,8,30,31]. Considering that 5 mM methyl-β-cyclodextrin depletes cholesterol from membranes [30,32], a ten-time lower concentration was used.

2.4. Insulin detection and secretion assays

For immunohistochemistry, pancreatic sections (8 μm) were deparaffinized and antigens retrieved (EDTA 10 mM pH 8, 96 °C/20 min). Sections were incubated with antibodies against insulin (ab7842, 1:100, Abcam, UK), and Alexa Fluor488-conjugated