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# Coenzyme $Q_{10}$ protects against β-cell toxicity induced by pravastatin treatment of hypercholesterolemia

Estela Lorza-Gil[1*] | Jane C. de Souza[1*] | Marta García-Arévalo[1] | Jean F. Vettorazzi[1] | Ana Carolina Marques[1] | Alessandro G. Salerno[1] | Jose Roberto Trigo[2,†] | Helena C. F. Oliveira[1]

[1]Department of Structural and Functional Biology, Biology Institute, State University of Campinas, Campinas, SP, Brazil

[2]Department of Animal Biology, Biology Institute, State University of Campinas, Campinas, SP, Brazil

**Correspondence**
Helena C. F. Oliveira, Lipid Metabolism Laboratory, Department of Structural and Functional Biology, Institute of Biology, State University of Campinas, Rua Monteiro Lobato, 255 Campinas, SP, CEP 13083-862, Brazil.
Email: ho98@g.unicamp.br

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## Abstract

New onset of diabetes is associated with the use of statins. We have recently demonstrated that pravastatin-treated hypercholesterolemic LDL receptor knockout ($LDLr^{-/-}$) mice exhibit reductions in insulin secretion and increased islet cell death and oxidative stress. Here, we hypothesized that these diabetogenic effects of pravastatin could be counteracted by treatment with the antioxidant coenzyme $Q_{10}$ ($CoQ_{10}$), an intermediate generated in the cholesterol synthesis pathway. $LDLr^{-/-}$ mice were treated with pravastatin and/or $CoQ_{10}$ for 2 months. Pravastatin treatment resulted in a 75% decrease of liver $CoQ_{10}$ content. Dietary $CoQ_{10}$ supplementation of pravastatin-treated mice reversed fasting hyperglycemia, improved glucose tolerance (20%) and insulin sensitivity (>2-fold), and fully restored islet glucose-stimulated insulin secretion impaired by pravastatin (40%). Pravastatin had no effect on insulin secretion of wild-type mice. In vitro, insulin-secreting INS1E cells cotreated with $CoQ_{10}$ were protected from cell death and oxidative stress induced by pravastatin. Simvastatin and atorvastatin were more potent in inducing dose-dependent INS1E cell death (10–15-fold), which were also attenuated by $CoQ_{10}$ cotreatment. Together, these results demonstrate that statins impair β-cell redox balance, function and viability. However, $CoQ_{10}$ supplementation can protect the statins detrimental effects on the endocrine pancreas.

**KEYWORDS**
β cell, cell death, coenzyme $Q_{10}$, insulin, statins

## 1 | INTRODUCTION

Statins are potent competitive inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, a microsomal enzyme that catalyzes the conversion of HMG-CoA to mevalonate, which is the rate-limiting step of the cholesterol synthesis pathway (Endo, 1992). Widely prescribed as cholesterol-lowering agents, statins are used as effective primary and secondary cardiovascular disease prevention, resulting in a significant reduction in cardiovascular morbidity and mortality (Gotto & Moon, 2013). In addition, statins exhibit several pleiotropic effects, including antioxidant and anti-inflammatory actions. Although statins are safe and well tolerated, some adverse effects affect 0.5–10% of treated patients, including increased levels of liver enzymes in plasma, muscle weakness, and myalgia (Minder,

*Estela Lorza-Gil and Jane C. de Souza contributed equally.

†In Memoriam.

  

Blumenthal, & Blaha, 2013). Novel formulations have been developed for sustained therapeutic effects and minimization of side effects (Clementino et al., 2016; Mathur & Kusum Devi, 2016).

Recent studies suggest that statins are also associated with an increased risk of developing type 2 diabetes (T2D; Betteridge & Carmena, 2016; Cederberg et al., 2015; N. A. Sattar et al., 2014). In March 2012, the US Food and Drug Administration (FDA) advised that statins can increase fasting blood glucose and glycated hemoglobin $A_{1c}$. Meta-analyses of large-scale statin trials supported the concept of a diabetogenic effect of statins, but the precise mechanisms involved have not been identified to date (Chan, Pang, & Watts, 2015; Thakker, Nair, Pagada, Jamdade, & Malik, 2016). We have recently revealed diabetes-related mechanisms induced by statin treatment in a familial hypercholesterolemia animal model (LDL receptor knockout mice). We demonstrated that chronic treatment with pravastatin induces pancreatic islet cell death associated with oxidative stress, resulting in decreased insulin secretion (Lorza-Gil et al., 2016).

By inhibiting HMG-CoA reductase, statins also inhibit other products of cholesterol biosynthetic pathway, such as coenzyme $Q_{10}$ ($CoQ_{10}$), dolichol and isoprenoids (Brault, Ray, Gomez, Mantzoros, & Daskalopoulou, 2014). $CoQ_{10}$ is a lipid-soluble molecule present in all cellular membranes and plasma lipoproteins (Bentinger, Tekle, & Dallner, 2010). $CoQ_{10}$ is a component of the mitochondrial electron transport chain and an abundant intracellular antioxidant that protects membrane components and low-density lipoproteins (LDL) from free radical-induced oxidative damage (Alleva et al., 1995). Several studies have demonstrated that treatment with different types of statins can lead to a parallel reduction in $CoQ_{10}$ and cholesterol in plasma (Folkers et al., 1990; Ghirlanda et al., 1993; Rundek, Naini, Sacco, Coates, & DiMauro, 2004) and tissues (Marcoff & Thompson, 2007). $CoQ_{10}$ deficiency has been implicated in statin-induced muscle mitochondrial dysfunctions (Busanello et al., 2017; La Guardia, Alberici, Ravagnani, Catharino, & Vercesi, 2013). In addition, low plasma $CoQ_{10}$ concentrations have been negatively correlated with poor glycemic control and diabetic complications (Ginter, 2014). Although it has been suggested that $CoQ_{10}$ deficiency impairs glucose metabolism (Chan et al., 2015), it is not clear whether supplementation of $CoQ_{10}$ prevents the development of T2D, especially in those with preexisting diabetic risk and receiving statin therapy.

We have recently demonstrated that pravastatin-treated LDL receptor knockout ($LDLr^{-/-}$) mice exhibit increased glycemia, marked reductions of fed insulinemia and glucose-stimulated insulin secretion by isolated pancreatic islets. These effects were associated with increased apoptosis indicators (Bax/Bcl2 protein ratio, cleaved caspase-3, TUNEL positive cells and lower NAD(P)H production rates) and oxidative stress (lipid peroxidation and $H_2O_2$ production; Lorza-Gil et al., 2016). Therefore, we proposed that the toxic effects of pravastatin on the hypercholesterolemic islets could be caused by the decrease in $CoQ_{10}$ biosynthesis, which would increase reactive oxygen species signaling to cell death. Thus, we investigated whether $CoQ_{10}$ supplementation restores the defective insulin secretion and cell death induced by pravastatin in $LDLr^{-/-}$ mice and insulin-secreting cells (INS1E).

## 2 | MATERIALS AND METHODS

### 2.1 | Animals and treatments

Low-density lipoprotein receptor knockout ($LDLr^{-/-}$; B6.129S7-*Ldlr*$^{tm1Her}$) female mice on the C57BL/6J background originally from the Jackson Laboratory (Bar Harbor, ME) were obtained from the breeding colony of the State University of Campinas (UNICAMP). Animal protocols were approved by the University's Committee for Ethics in Animal Experimentation (Comissão de Ética no Uso de Animais da UNICAMP, CEUA/UNICAMP, protocol #3819-1) and all experiments were performed in accordance with relevant guidelines and regulations. The mice had free access to regular rodent AIN93-M diet (14% protein) and water and were housed at 22 ± 1°C on a 12-hr light/dark cycle. Female mice (4 weeks old) were treated with pravastatin dissolved in the drinking water (400 mg/L) and/or chow containing $CoQ_{10}$ (3 g/kg diet) for 2 months. The pravastatin sodium (Medley Farmacêutica Ltda, Campinas, SP, Brazil) dose of 40 mg/kg body weight (bw) per day was based on the drink consumption rate measurement (3 ml/day). The $CoQ_{10}$ (Shenzhou Biology & Technology, Huhehot-Inner, Mongolia, China) dose of 300 mg/kg bw per day was based on the food consumption rate measurement (3 g/day). We chose to study female $LDLr^{-/-}$ mice for several reasons, including increased susceptibility to atherosclerosis, higher plasma cholesterol levels and increased response to statin treatment compared with males. In addition, recent reports on diabetogenic effects of statins demonstrate an increased risk for women compared with men.

### 2.2 | Plasma biochemical analyses

Blood glucose was measured using a glucose analyzer (Accu-Chek Advantage; Roche Diagnostics, Basel, Switzerland). Plasma cholesterol, triglycerides and enzymes, including aspartate transaminase (AST) and alanine transaminase (ALT), were measured using standard commercial kits (Roche Diagnostics GMbH, Mannheim, Germany), for cholesterol and triglycerides, and Bioclin (Belo Horizonte, Brazil, for transaminases) according to the manufacturer's instructions.

### 2.3 | Oral glucose tolerance test (GTT) and insulin tolerance test (ITT)

After 12 hr of fasting, mice received an oral dose of glucose solution (1.5 g/kg bw). Blood samples were collected from the tail tip before the oral dose ($t$ = 0 min) and at 15, 30, 60, 90, and 120 min after glucose administration. For the ITT, blood was taken from mice that had been fasted overnight and refed for 3 hr ($t$ = 0 min) and at 5, 10, 15, 30, and 60 min after an intraperitoneal insulin injection (0.75 U/kg bw, regular human insulin; Eli Lilly Company, Indianapolis, IN) for glucose analysis.

### 2.4 | Liver levels of $CoQ_{10}$

The extraction procedure for $CoQ_{10}$ from mouse tissues was adapted from Tang et al. (2004). Pieces of liver were accurately weighed in the

frozen state (−80°C) and subsequently homogenized with 1.5 ml of cold 1-propanol in a Tissue Lyser LT (Qiagen, Hilden, Germany). Then, 100 µl of cold water was added to the homogenized mixture, which was vortex-mixed for 20 s. The mixture was centrifuged at 10,000 rpm for 5 min at 4°C. The clear supernatant was immediately transferred to a polypropylene tube and dried using a speed vacuum (Eppenforf, Hamburg, Germany). The extract was resuspended in 100 µl of cold 1-propanol for $CoQ_{10}$ quantification. $CoQ_{10}$ quantification was performed by high performance liquid chromatography (HPLC)-diode array detector (DAD). A 50 µl sample was analyzed in an HPLC Agilent 1260 Infinity Quaternary Pump equipped with a Standard Autosampler and a Diode Array-Multiple Wavelength Detector. The analytical column was a reversed-phase Eclipse Plus C18 (4.6 × 250 mm, 5 µm) maintained at 30°C. The mobile phase consisted of an isocratic mixture of 1:1 methanol:isopropanol. The flow rate was 1 ml/min for 30 min. The detector was set at 275 nm. The reference calibration curve (0.1–3.6 µg/ml) was prepared using $CoQ_{10}$ (Sigma-Aldrich, Saint Louis, MO) dissolved in 1:1 methanol:isopropanol.

## 2.5 | Pancreatic islet isolation and static insulin secretion

Pancreatic islets were isolated from fasted mice by collagenase type V (0.8 mg/ml; Sigma) digestion and selected with a microscope (Boschero & Delattre, 1985). Quadruplicates of each condition (2.8 and 11.1 mM glucose) containing four islets per well from each mouse ($n$ = 4–8 mice per group) were used for the insulin secretion assay. Islets were preincubated for 30 min at 37°C in Krebs-bicarbonate buffer (KBB) of the following composition: 115 mmol/L NaCl, 5 mmol/L KCl, 2.56 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, 10 mmol/L $NaHCO_3$, 15 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), supplemented with 5.6 mmol/L glucose and 0.3% bovine serum albumin (BSA), equilibrated with a mixture of 95% $O_2$/5% $CO_2$, pH 7.4. The islets were further incubated for 1 hr in KBB containing glucose (2.8 or 11.1 mmol/L). At the end of the incubation period, insulin secreted into the media was measured by radioimmunoassay (Scott, Atwater, & Rojas, 1981).

## 2.6 | Pancreatic islet ex vivo treatment

Pools of 100 islets of $LDLr^{-/-}$ female mice manually collected under the laminar flow were plated and maintained for 16 hr in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 5.6 mM glucose and 10% fetal bovine serum (FBS) in a humidified atmosphere at 37°C and 5% $CO_2$. After this period, pancreatic islets were incubated in RPMI 1640 medium supplemented with 5.6 mM glucose, 1% FBS, and 5% BSA in the presence of 70 µM pravastatin (Sigma) and/or 10 µM $CoQ_{10}$ (Sigma) for 48 hr. Subsequently, pancreatic islets were used for insulin secretion assay or western blot. For the Western blot assay, islets were homogenized in urea lysis buffer (7 mM urea and 2 mM thiourea) containing protease inhibitors.

## 2.7 | INS-1E cell culture

INS-1E cells (kindly provided by Décio L. Eizirik, Center for Diabetes Research, Universite Libre de Bruxells) were cultured in a humidified atmosphere containing 5% $CO_2$ and maintained in RPMI 1640 medium (11 mM glucose, 5% FBS, 1% HEPES, 1% sodium pyruvate, 0.1% β-mercaptoethanol, and 1% penicillin/streptomycin) until 60–80% confluence. Cells were incubated with different classes of statins: pravastatin sodium salt 1–50 µM (Sigma; P4498); simvastatin 1–5 µM (Sigma; S6196); atorvastatin 1–50 µM (Sigma; Z0001) and/or 5–10 µM $CoQ_{10}$ (Sigma; C9538) for 12–48 hr. Dimethyl sulfoxide (DMSO; Sigma; D8418) was used as vehicle for lipophilic statins (simvastatin and atorvastatin). Cyclopiazonic acid (CPA) 15 µM (Sigma; C1530) was used as cell death positive control. Control and treated cells were used for $H_2O_2$ (Amplex-red, Molecular Probes, Eugene, OR) and cell viability measurements (Hoechst plus propidium iodide [PI] staining). Additionally, cells were lysed and homogenized in radioimmunoprecipitation assay buffer plus protease inhibitors for Western blot analyses.

## 2.8 | Western blot analysis

Cell or islets homogenate were treated with Laemmli loading buffer containing dithiothreitol. After heating to 95°C for 5 min, the proteins were separated by electrophoresis (30 µg protein/lane, 12% acrylamide/bisacrylamide gel) and transferred to nitrocellulose membranes. The nitrocellulose membranes were treated for 1.5 hr with a blocking buffer (5% BSA, 10 mmol/L Tris, 150 mmol/L NaCl, and 0.02% Tween 20). Membranes were cut into two pieces according to molecular weight markers, to proceed in parallel with the detection of internal controls (glyceraldehyde 3-phosphate dehydrogenase [GAPDH] or tubulin) and target protein (cleaved caspase-3). Membranes were then incubated with the primary rabbit antibody against caspase-3 (Millipore Corporation, Billerica, MA, AB3623), with rabbit polyclonal antibody against GAPDH (Santa Cruz Biotechnology, Inc., Dallas, TX, SC-2577-8) and with mouse monoclonal antibody against tubulin (SC-5286). Subsequently, membranes were then incubated with horseradish peroxidase-conjugated secondary antibody (1:10,000; Invitrogen, Waltham, MA). Detection was performed using enhanced chemiluminescence (SuperSignal West Pico, Pierce, Rockford, IL). Band intensities were quantified by optical densitometry (Scion Image, Frederick, MD).

## 2.9 | Assessment of cell death

INS1E cells ($10^4$ cells per well) were incubated in a 96-well plate with statins and/or $CoQ_{10}$ (Sigma) for 24–48 hr. The percentage of dead cells were determined after a 15-min incubation with DNA-binding dyes Hoechst (HO; Sigma; H33342; 5 µg/ml) and PI (PI; Invitrogen P1304; 5 µg/ml; Marroqui et al., 2014). Images were obtained using the FLoid® Cell Imaging Station (Thermo-Fischer, Waltham, MA). HO freely passes the plasma membrane and stains DNA blue, whereas PI, a highly polar dye that is impermeable to cells with intact membranes, stains the DNA of dead cells red. The percentage of cell death (PI/HO × 100) was

quantified using Image J software (Image J- Bethesda, MD). The results are expressed as average of three independent assays, using in each assay 3–4 replicates of each condition.

## 2.10 | Cell viability assay

INS-1E cells were seeded in 96 wells per plate. After 48 hr incubation, with different statin classes, the cells were washed with PBS and then 100 μl of RPMI-1640 without phenol-red was added in each well to assess the cell viability using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, MTT was dissolved in PBS at a concentration of 5 mg/ml. MTT was then added to each well (10 μl per 100 μl medium) and plates were incubated at 37°C for 3 hr in the absence of light. The medium was replaced with 100 μl of MTT solubilization solution (Sigma; M8910). The plate was kept in a shaker for 1 hr and then the absorbance for each well was measured at 570 nm using a Spectramax™ microplate spectrophotometer (Molecular Devices, Sunnyvale, CA).

## 2.11 | INS-1E cell $H_2O_2$ production

$H_2O_2$ release was monitored by measuring the conversion of Amplex Red to highly fluorescent resorufin in the presence of added horseradish peroxidase. Briefly, $5 \times 10^4$ cells were incubated in 96-well cell culture plates with pravastatin and/or $CoQ_{10}$ (Sigma) for 24 hr. On the day of the experiment, cells were incubated in a mixture containing 50 μM Amplex Red reagent (Invitrogen; A22188) and 0.1 U/ml horseradish peroxidase in Krebs-Ringer phosphate buffer (145 mM NaCl, 5.7 mM sodium phosphate, 4.86 mM KCl, 0.54 mM $CaCl_2$, 1.22 mM $MgSO_4$, 11.1 mM glucose, pH 7.35). This assay was conducted in the presence and absence of catalase (500 U/ml) for 1 hr. Fluorescence was monitored over time in a temperature-controlled SpectraMax M3 Microplate Reader (Molecular Devices) using excitation and emission wavelengths of 560 and 590 nm, respectively.

## 2.12 | Pancreatic islet aconitase activity

Aconitase activity, a Krebs cycle enzyme highly sensitive to superoxide, was determined by the method described by Morrison (1954), using a medium containing 50 mM $KH_2PO_4$, 10% triton X-100, 0.07 mM sodium citrate, 1.3 mM manganese chloride. The reduction of $NADP^+$ was measured at the wavelengths 340 (excitation) and 466 nm (emission) and expressed as mU/mg protein (1 U = 1 μmol NADPH/min/mg protein).

## 2.13 | Statistical analyses

The data are presented as the mean ± standard error (SE; *n* is indicated in each figure). The groups were compared using one-way analysis of variance (ANOVA) with LSD (Fisher's least significance difference) post hoc test (Graphpad Prism, RRID: SCR_002798; URL: www.graphpad.com). In some experiments (indicated in the figure legends), a *t*-test was performed to compare untreated versus pravastatin treatment. The level of significance was set at $p \leq 0.05$.

# 3 | RESULTS

Previous studies have shown that, in addition to decreasing cholesterol levels, statins treatment reduces the levels of $CoQ_{10}$. Given that the liver is the major steroidogenic organ and the major target of statins, we measured the liver $CoQ_{10}$ content of $LDLr^{-/-}$ mice treated or not with pravastatin. Pravastatin treatment induced a 75% decrease in $CoQ_{10}$ hepatic levels of $LDLr^{-/-}$ mice compared with the untreated group, while the groups of $LDLr^{-/-}$ mice supplemented with $CoQ_{10}$ in the diet (300 mg/kg bw) had increased $CoQ_{10}$ hepatic levels by about 20-fold (Figure 1a). In addition, we determined the levels of $CoQ_{10}$ in the plasma and found a marked elevation in $CoQ_{10}$ concentrations in the supplemented groups (Figure 1b). Body weight and blood levels of glucose, lipids, and liver enzymes of treated mice are presented in Table 1. No significant changes were observed in body weight and plasma triglycerides

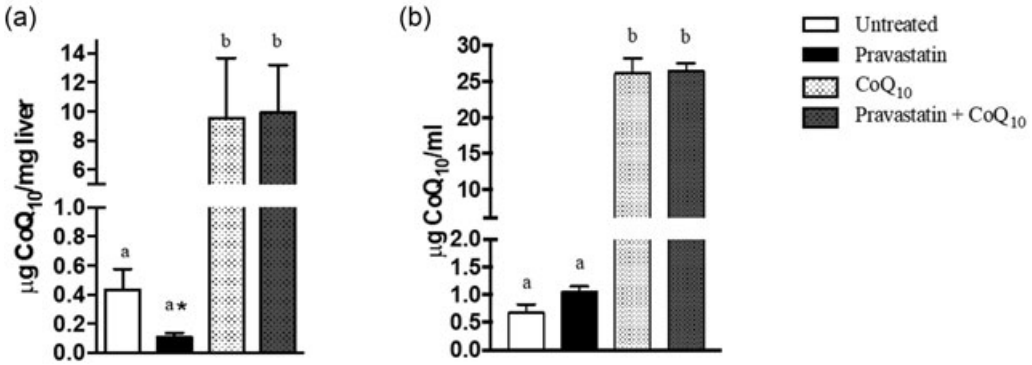


**FIGURE 1** Plasma and hepatic $CoQ_{10}$ levels in pravastatin-treated mice. Female $LDLr^{-/-}$ mice were treated with pravastatin (40 mg/kg bw) and/or $CoQ_{10}$ (300 mg/kg bw) for 2 months. Liver (a) and plasma (b) $CoQ_{10}$ concentrations were determined by HPLC with a diode array detector. Mean ± *SE* (*n* = 7–8 for liver and *n* = 3–4 for plasma). Different letters represent significant differences ($p < 0.05$) between groups by one-way ANOVA with least significant difference (LSD) post hoc test. *$p < 0.05$ by Student's *t*-test between untreated versus pravastatin-treated mice. ANOVA: analysis of variance; bw: body weight; $CoQ_{10}$: coenzyme $Q_{10}$; HPLC: high performance liquid chromatography; LSD: least significant difference

**TABLE 1** Body weight and fasting blood levels of glucose, lipids and liver enzymes of LDLr$^{-/-}$ mice treated with pravastatin and/or $CoQ_{10}$ for 2 months

| | Untreated | Pravastatin | $CoQ_{10}$ | Pravastatin + $CoQ_{10}$ |
|---|---|---|---|---|
| Body weight (g) | 20.6 ± 0.73 | 21.3 ± 0.47 | 20.8 ± 0.66 | 21.1 ± 0.47 |
| Glucose (mg/dl) | 81.9 ± 3.45[a] | 96.9 ± 6.28[b] | 81.9 ± 4.32[a] | 75.1 ± 4.48[a] |
| TG (mg/dl) | 159.2 ± 27.35 | 120.0 ± 11.59 | 148.0 ± 10.69 | 105.9 ± 6.54 |
| CHOL (mg/dl) | 422.7 ± 36.97[a] | 277.9 ± 16.60[b] | 409.4 ± 19.20[a] | 262.0 ± 27.94[b] |
| ALT (U/L) | 29.4 ± 9.55 | 40.3 ± 9.34 | 32.4 ± 7.88 | 33.9 ± 5.94 |
| AST (U/L) | 28.1 ± 8.24 | 17.2 ± 7.95 | 26.0 ± 8.98 | 26.0 ± 9.82 |

*Note.* Mean ± SE, n = 8. Different letters represent significant differences ($p < 0.05$) between groups by one-way ANOVA with LSD post hoc test. ALT: alanine transaminase; ANOVA: analysis of variance; AST: aspartate transaminase; CHOL: cholesterol; $CoQ_{10}$: coenzyme $Q_{10}$; LSD: least significant difference; TG: triglyceride.

among the groups. As expected, pravastatin treatment of 2 months reduced total plasma cholesterol levels by 36% in LDLr$^{-/-}$ mice with or without $CoQ_{10}$ supplementation. Pravastatin treatment increased (18%) fasting plasma glucose levels, and the combined treatment of $CoQ_{10}$ with pravastatin reversed this hyperglycemia to normal levels. No alterations in plasma transaminases levels (ALT or AST) were observed, excluding liver hepatotoxicity during the treatments.

### 3.1 | $CoQ_{10}$ diet supplementation improves glucose tolerance and insulin sensitivity and normalizes insulin secretion in pravastatin-treated LDLr$^{-/-}$ mice

The effects of $CoQ_{10}$ diet supplementation on glucose homeostasis and pancreatic insulin secretion are presented in Figures 2 and 3, respectively. As previously demonstrated (Lorza-Gil et al., 2016), pravastatin treatment of 2 months did not affect glucose tolerance. However, $CoQ_{10}$ diet supplementation per se (combined or not with pravastatin) significantly improved glucose tolerance of hypercholesterolemic mice in response to the glucose oral load (Figure 2a). This finding is verified by the reduced area under the glycemic curve (Figure 2b). Global insulin resistance was evaluated by insulin tolerance test (ITT). After insulin administration, LDLr$^{-/-}$ mice supplemented with $CoQ_{10}$ exhibited increased blood glucose disappearance rates during the first 10 min ($K_{ITT}$) indicating an enhanced insulin sensitivity (Figure 2c,d). This finding explains the improved glucose tolerance of mice treated with $CoQ_{10}$. Next, we evaluated insulin secretion by isolated pancreatic islets after glucose stimulation (11.1 mM) and demonstrated that the reduc-

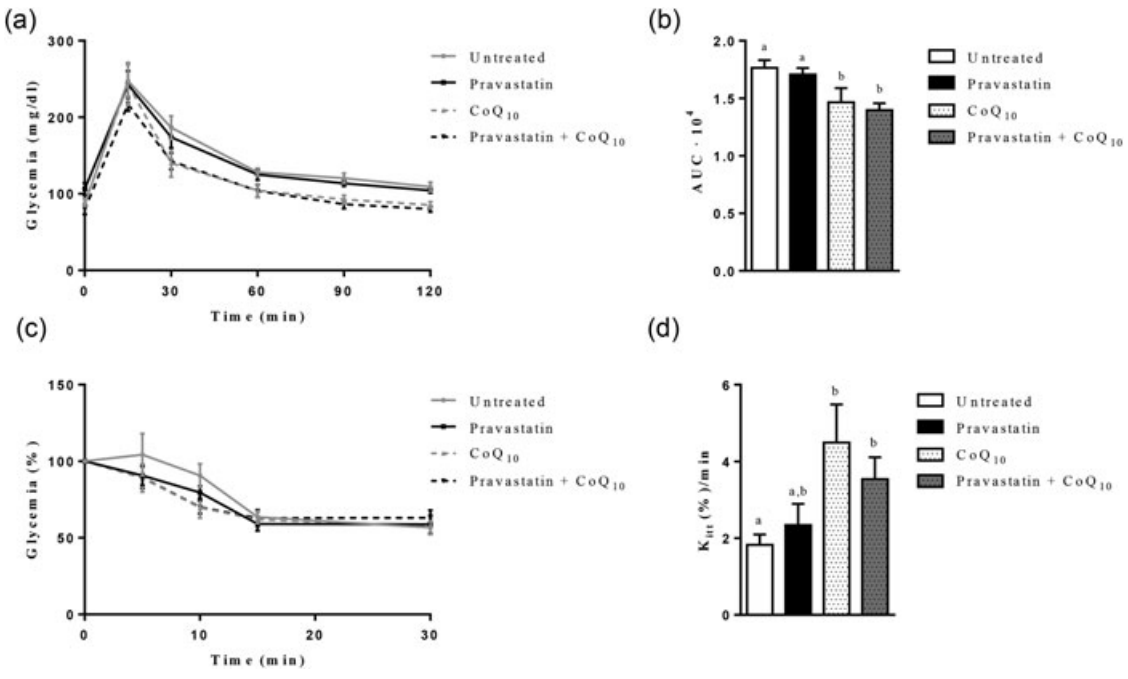


**FIGURE 2** $CoQ_{10}$ diet supplementation improves glucose tolerance and insulin sensitivity in LDLr$^{-/-}$ mice. GTT (a) and ITT (c) were performed after 2 months of pravastatin and/or $CoQ_{10}$ treatment in LDLr$^{-/-}$ mice. (a) Blood glucose concentrations and (b) areas under the glycemic curves during the GTT. (c) Blood glucose concentrations (%) and (d) disappearance rate ($K_{ITT}$) during the first 10 min of ITT. Mean ± *SE* ($n = 5–7$). Different letters represent significant differences ($p < 0.05$) between groups by one-way ANOVA with LSD post hoc test. ANOVA: analysis of variance; $CoQ_{10}$: coenzyme $Q_{10}$; GTT: glucose tolerance test; ITT: insulin tolerance test

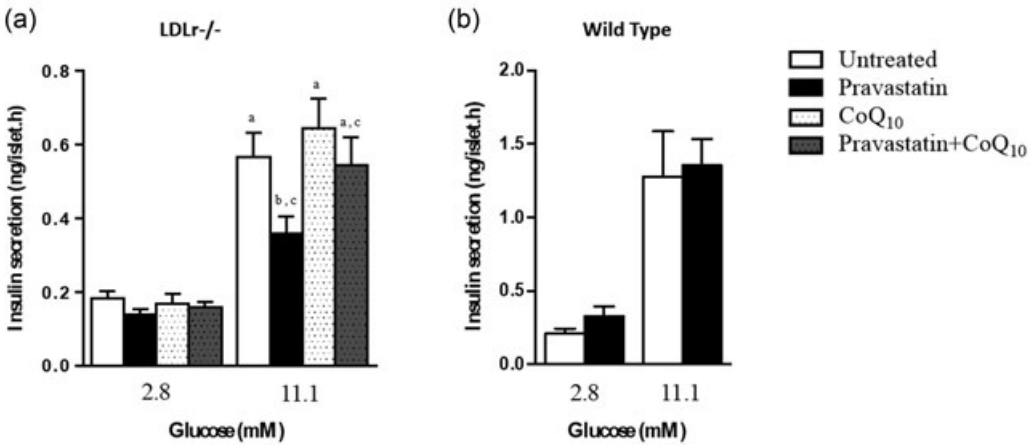


**FIGURE 3** Impairment of glucose-stimulated insulin secretion in pravastatin-treated LDLr$^{-/-}$ mice is prevented by $CoQ_{10}$ diet supplementation. Insulin secretion from isolated islets of LDLr$^{-/-}$ (a) or wild-type (b) mice after 2 months of pravastatin and/or $CoQ_{10}$ treatment. The islets were incubated with 2.8 and 11.1 mM glucose. Mean ± *SE* ($n$ = 12). Different letters represent significant differences ($p < 0.05$) between groups by one-way ANOVA with LSD post hoc test. ANOVA: analysis of variance; $CoQ_{10}$: coenzyme $Q_{10}$; LSD: least significant difference

tion in insulin secretion induced by pravastatin treatment (40%) was fully reversed to normal levels in mice supplemented with $CoQ_{10}$ (Figure 3a).

Since these LDLr$^{-/-}$ mice exhibit a prediabetic phenotype, we further evaluated the effect of pravastatin in control normolipidemic wild-type C57BL6/J mice. We found no changes in glucose stimulated insulin secretion rates by isolated islets of pravastatin-treated wild-type mice (Figure 3b). In addition, pravastatin did not alter the glucose tolerance and plasma levels of glucose and insulin of wild-type mice (data not shown). These results suggest that the cholesterol metabolism defect is responsible for the increased sensitivity of islets to pravastatin that is not observed in islets from C57BL6/J wild-type background.

## 3.2 | $CoQ_{10}$ protects isolated islets from pravastatin-induced defective insulin secretion and cell death

Our previous in vivo study showed that 2 months of pravastatin treatment reduced glucose-stimulated insulin secretion and increased apoptosis signaling and cell death in islets from treated LDLr$^{-/-}$ mice (Lorza-Gil et al., 2016). Here, we demonstrate that these in vivo results are reproduced in short term ex vivo experiments with LDLr$^{-/-}$ islets incubated (48 hr) with pravastatin (70 μM). In addition, these adverse effects were reversed by the presence of $CoQ_{10}$ (10 μM; Figure 4). Figure 4a shows that the reduction in glucose-stimulated insulin secretion induced by pravastatin (30%) was prevented by coincubation of islets with $CoQ_{10}$. Figure 4b shows that pravastatin increased the activation of the final apoptosis effector (cleaved caspase-3) by approximately 50%, which was prevented by coincubation with $CoQ_{10}$.

## 3.3 | $CoQ_{10}$ protects insulin-secreting INS1E cells against statins-induced cell death

We previously demonstrated that pravastatin induced time- and dose-dependent apoptosis indicated by caspase-3 activation in insulin-secreting INS-1E cells (Lorza-Gil et al., 2016). Here, we investigated cell death (necrosis) using propidium iodide DNA staining. Figure 5 demonstrates that 24 hr of incubation with 10 μM of pravastatin resulted in a significant increase in the amount of cell death (32%). Coincubation with 10 μM of $CoQ_{10}$ for 24 hr significantly decreased the percentage of dead INS1E cells caused by pravastatin treatment (Figure 5a,b). The protective effects of $CoQ_{10}$ in pravastatin-treated cells were also confirmed by measuring the protein levels of cleaved caspase-3 in INS1E cells cotreated with $CoQ_{10}$ for 24 hr. The 50% increase in caspase-3 activation in pravastatin-treated cells was attenuated by the presence of $CoQ_{10}$ (Figure 5c,d).

To confirm that these effects were not restricted to pravastatin, we treated INS1E cells with two other statins, simvastatin and atorvastatin. In Figure 6a, and b, we show that incubations with simvastatin (0–5 μM) and with atorvastatin (0–50 μM), respectively, induce dose-dependent cell death, much more potently (10–15-fold above the zero dose) than that observed with pravastatin. $CoQ_{10}$ decreased simvastatin effect by 60% ($p < 0.001$) and the atorvastatin effect by 30% ($p < 0.05$). Interestingly, incubation with $CoQ_{10}$ alone also induced cell death in doses ≥ 10 μM (Figure 6c). The possible reasons to explain this effect are at least two: (a) antioxidant supplements may turn prooxidative at high concentrations, and (b) their capacity to scavenge ROS normally present at physiological conditions that are required for optimal cellular functioning (Bouayed & Bohn, 2010). Nonetheless, together with statins, the $CoQ_{10}$ effect is always protective. The $CoQ_{10}$ also had a mild but significant protective effect against CPA, a positive

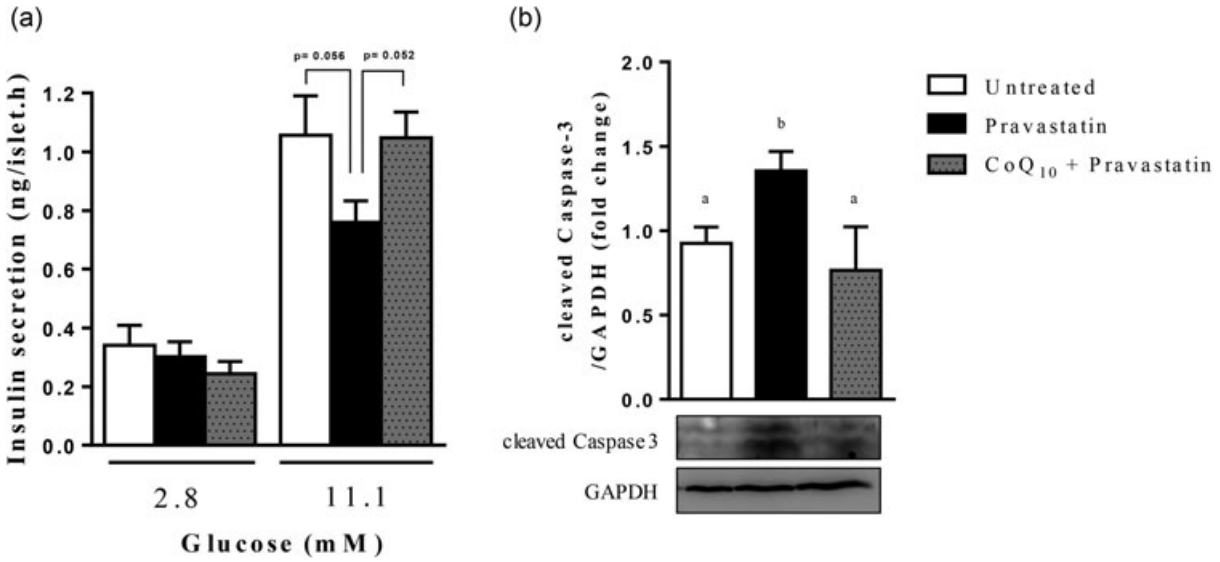


**FIGURE 4** Ex vivo $CoQ_{10}$ treatment protects $LDLr^{-/-}$ isolated islets from pravastatin-induced β-cell dysfunction and cell death. Glucose stimulated insulin secretion (a) and cleaved caspase-3 content (b, western blot analysis) in isolated islets treated with pravastatin (70 μM) and/or $CoQ_{10}$ (10 μM) for 48 hr. Mean ± *SE* ($n$ = 6–9 mice, three independent experiments). Different letters represent significant differences ($p < 0.05$) between groups by one-way ANOVA with LSD post hoc test. ANOVA: analysis of variance; $CoQ_{10}$: coenzyme $Q_{10}$; LSD: least significant difference

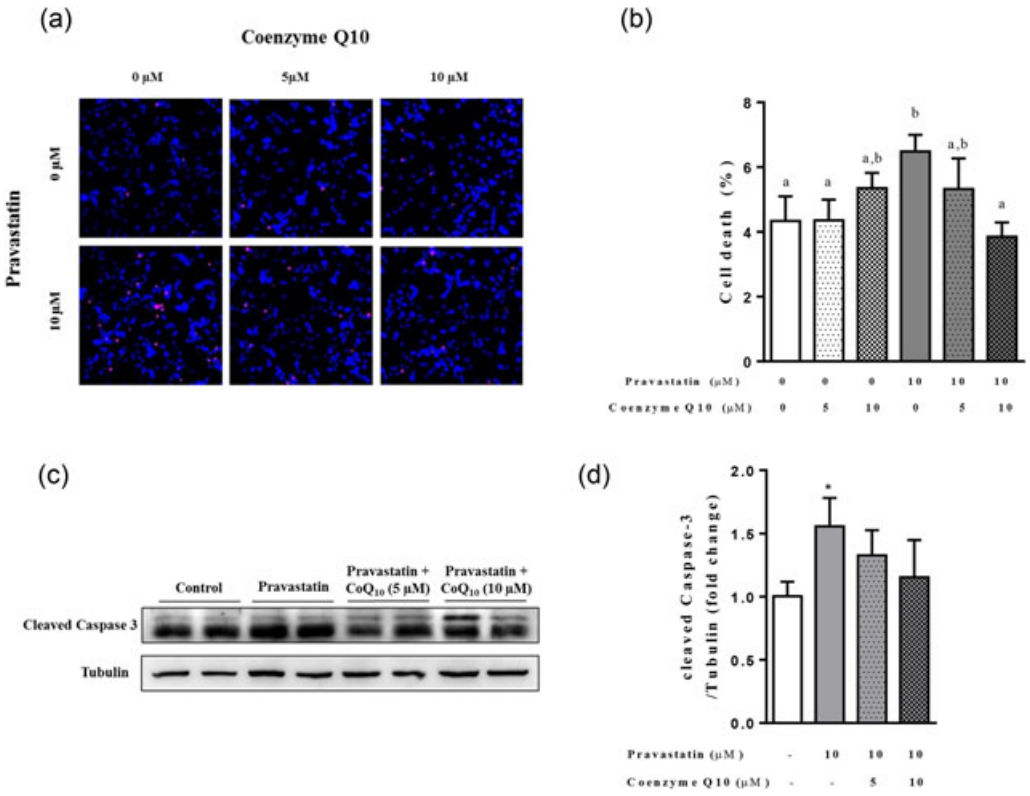


**FIGURE 5** $CoQ_{10}$ protects against cell death induced by pravastatin treatment of INS1E cells. (a) Representative images of INS1E cells stained with Hoechst in blue (HO) and PI in red after 10 μM pravastatin and /or $CoQ_{10}$ (5 or 10 μM) treatment of 24 hr and (b) quantification of INS1E cell death, indicating the percentage of dead cells (PI) in relation to total number of cells (HO). Mean ± *SE* (six replicates in three independent experiments). (c) Representative western blot bands and (d) quantification of cleaved caspase 3 in INS1E cells treated with pravastatin (10 μM) and $CoQ_{10}$ (5 or 10 μM). Mean ± *SE* ($n$ = 6, three independent experiments). Different letters represent significant differences ($p < 0.05$) between groups by one-way ANOVA with LSD post hoc test. *$p < 0.05$ by Student's $t$-test between untreated versus pravastatin-treated cells. ANOVA: analysis of variance; $CoQ_{10}$: coenzyme $Q_{10}$; PI: propidium iodide; LSD: least significant difference
[Color figure can be viewed at wileyonlinelibrary.com]

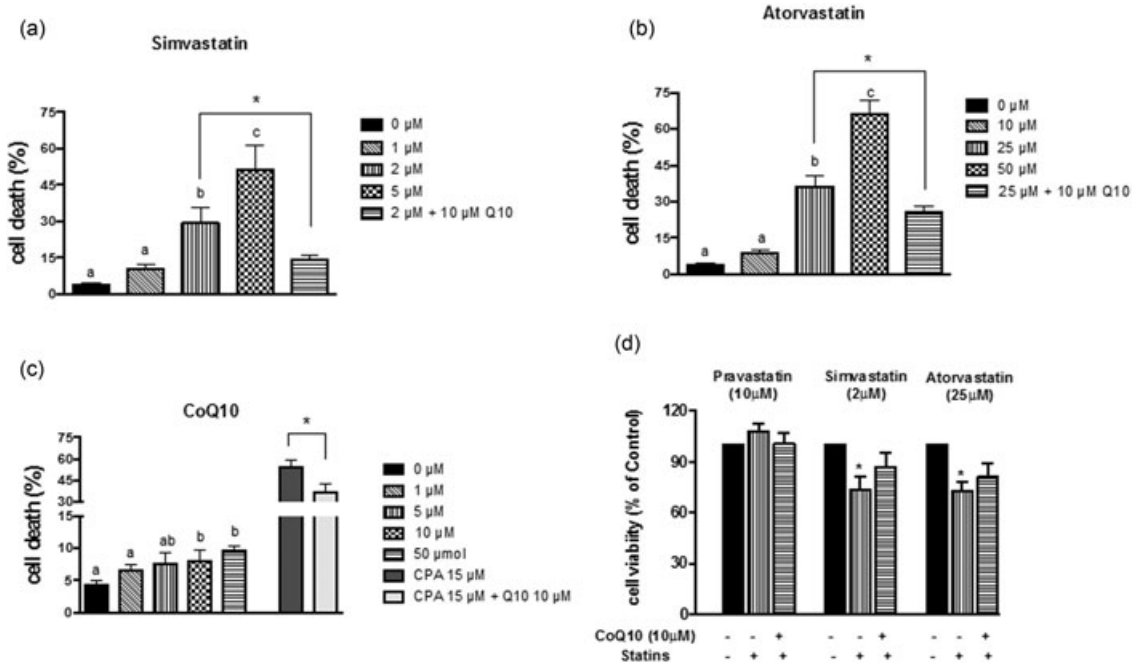


**FIGURE 6** $CoQ_{10}$ protects against cell death induced by simvastatin and atorvastatin treatment of INS1E cells. Quantification of INS1E cell death by HO and PI staining after treatments with simvastatin (0–5 µM) (a), atorvastatin (0–50 µM) (b), $CoQ_{10}$ (0–50 µM) (c) for 48 hr, and cell viability (MTT) after statins incubations (d). DMSO was used as vehicle for simvastatin and atorvastatin. CPA (cyclopiazonic acid) was used as a cell death positive control. Mean ± *SE* (14 replicates in five independent experiments). Different letters represent significant differences ($p < 0.05$) between groups by one-way ANOVA with LSD post hoc test. *$p < 0.001$ for simvastatin and $p < 0.05$ for atorvastatin and CPA by Student's *t*-test. ANOVA: analysis of variance; $CoQ_{10}$: coenzyme $Q_{10}$; HO: Hoechst; LSD: least significant difference; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PI: propidium iodide

control for cell death (Figure 6c). CPA induces cell death by blocking SERCA and causing ER stress (Pirot et al., 2007).

Since pravastatin caused much less cell death in INS1E cells than simvastatin and atorvastatin, we also determined the effects of statins on cell viability (Figure 6d) using the MTT technique. Accordingly, pravastatin had no effect and simvastatin and atorvastatin decreased significantly INS1E viability, effects that were attenuated by coincubation with $CoQ_{10}$. These results reinforce the interpretation that pravastatin toxic effects occurs only in the hypercholesterolemic context, as shown in Figure 3a ($LDLr^{-/-}$) compared with Figure 3b (wild-type). Thus, INS1E cells are limited as a model for the hypercholesterolemic β cells, but are useful for verifying protective effects of $CoQ_{10}$, particularly for those more potent statins.

### 3.4 | $CoQ_{10}$ protects INS1E cells against statin-induced $H_2O_2$ production

In the previous study, we demonstrated that increased apoptosis in pravastatin-treated INS1E cells was associated with an increase of hydrogen peroxide net production rate. Given that $CoQ_{10}$ functions as an important cell antioxidant, we hypothesized that $CoQ_{10}$ could reduce the enhanced $H_2O_2$ production induced by pravastatin. In Figure 7, we demonstrate that hydrogen peroxide production is significantly increased in 10 µM pravastatin (Figure 7a,b, 20% vs. control) and 2 µM simvastatin (Figure 7c,d, 33% vs. control) treated cells. In the presence of $CoQ_{10}$, hydrogen peroxide production is greatly reduced to levels even lower than the control (pravastatin) or to control levels (simvastatin). Catalase addition was done to show specificity of the assay by inhibiting of amplex red fluorescence.

### 3.5 | $CoQ_{10}$ diet supplementation prevents pravastatin induced inhibition of aconitase in islets of treated $LDLr^{-/-}$ mice

The above results led to the hypothesis that the oxidative stress is the main statin induced toxic mechanism for islet or β cell. To confirm this we should be able to show a key oxidized target. Thus, we measured the activity of an enzyme that is very sensitive to superoxide production and is also an indicative of mitochondrial redox state: the Krebs cycle enzyme aconitase. Indeed, we found that pravastatin-treated $LDLr^{-/-}$ mice islet had significantly decreased aconitase activity (40%, Figure 8) and $CoQ_{10}$ cotreatment fully prevent this effect. Interestingly, $CoQ_{10}$ treatment alone increased aconitase activity in islets above the control levels, showing that $CoQ_{10}$ supplementation scavenges basal levels of superoxide.

## 4 | DISCUSSION

Statins are often used to treat genetic hypercholesterolemic patients. Thus, we chose the appropriate in vivo metabolic context, namely, the mouse model that mimics the human disease familial hyperch-

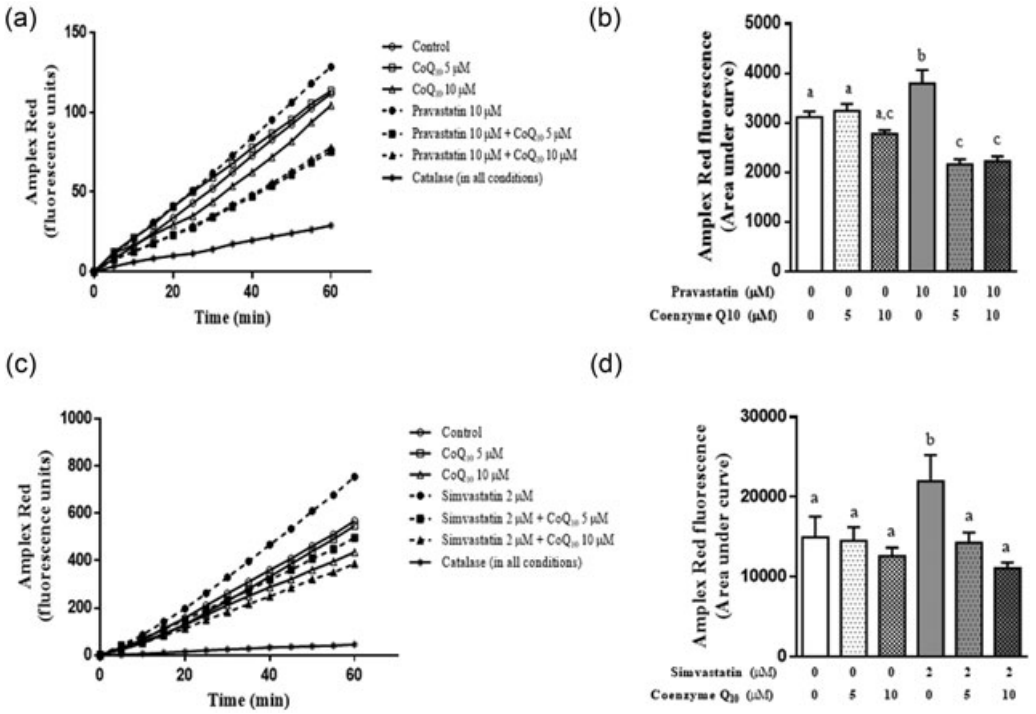


**FIGURE 7** $CoQ_{10}$ protects against $H_2O_2$ production induced by statins in INS1E cells. $H_2O_2$ generation in INS1E cells treated with pravastatin (a,b) and simvastatin (c,d) and/or $CoQ_{10}$ was determined by the Amplex red derived fluorescence. (a,c) Fluorescence curves after treatments with pravastatin (10 µM), simvastatin (2 µM) and/or $CoQ_{10}$ (5 or 10 µM). (b,d) Total area under the curves of Amplex Red fluorescence. Mean ± *SE* (six replicates in three independent experiments). Different letters represent significant differences ($p < 0.05$) between groups by one-way ANOVA with LSD post hoc test. ANOVA: analysis of variance; $CoQ_{10}$: coenzyme $Q_{10}$; LSD: least significant difference

olesterolemia, to study prevention of statin diabetogenic effects. We recently identified pravastatin toxic mechanisms responsible for disturbed glucose homeostasis in female $LDLr^{-/-}$ mice. These pravastatin treated mice presented hyperglycemia and hypoinsulinemia in the fed state and marked reductions of glucose-stimulated pancreatic islet insulin secretion. These effects were associated with islet oxidative stress ($H_2O_2$ overproduction) and damage (lipoperoxidation) leading to cell death (Lorza-Gil et al., 2016). In the present work, we tested the hypothesis that $CoQ_{10}$ supplementation could prevent these diabetogenic effects of pravastatin. We demonstrate that pravastatin chronic treatment causes liver depletion of $CoQ_{10}$, a major intracellular antioxidant, which production is also inhibited by statins. $CoQ_{10}$ supplementation in vivo, ex vivo, and in vitro prevented or attenuated the toxic statins effects. $CoQ_{10}$ reversed hyperglycemia, improved glucose tolerance and insulin sensitivity, restored glucose stimulated insulin secretion, and protected islet and β cell from oxidative stress and death.

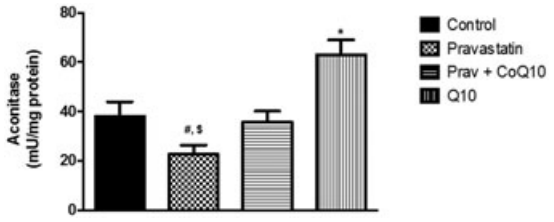


**FIGURE 8** Chronic treatment with pravastatin decreases aconitase activity in pancreatic islets and $CoQ_{10}$ diet supplementation reverses this effect. Aconitase activity was evaluated in isolated islets after 2 months of pravastatin and/or $CoQ_{10}$ treatment of $LDLr^{-/-}$ mice. Mean ± *SE* ($n = 4–6$). ANOVA with LSD post hoc test: $^{*}p < 0.05$ versus all groups and $^{\#}p = 0.07$ versus control; Student's *t*-test: $^{\$}p < 0.05$ versus control. ANOVA: analysis of variance; $CoQ_{10}$: coenzyme $Q_{10}$; LSD: least significant difference

Specific populations exhibit an increased risk for developing diabetes during statin therapy, such as the elderly (age > 70 years), those of Asian ethnicity and women (Culver et al., 2012; N. A. Sattar et al., 2014; Sonal Sekhar & Unnikrishnan, 2015). Sex differences have been reported only recently, with increased risk of diabetes in women on statins, particularly in postmenopausal women (Culver et al., 2012). Interestingly, the frequency of onset of diabetes in statins trials increases in parallel with increasing women participation in these trials (Aiman, Najmi, & Khan, 2014; Goodarzi, Li, Krauss, Rotter, & Chen, 2013). This finding seems to suggest a sex-specific drug response and not disease susceptibility, given that diabetes prevalence in the general population is increased in men (9.8%) compared with women (9.2%; Danaei et al., 2011; N. Sattar, 2013).

The diabetogenic effects of statins may vary with the dose and degree of hydrophobicity of statins. A meta-analysis by Preiss et al., including 5 types of statins trials and 32,752 participants without pre-existing diabetes, confirmed that statins have a diabetogenic dose-dependent effect with a 12% increased risk for high-dose

therapy (40–80 mg/day) compared with moderate doses (10–40 mg/day; Preiss et al., 2011). Lipophilic statins, such as atorvastatin and simvastatin, which decrease cholesterol more potently, are also more diabetogenic than the hydrophilic statins, such as pravastatin (Carter et al., 2013). This is in agreement with data presented here. It has been suggested that many of the adverse effects of statins in organs other than pancreas may be related to $CoQ_{10}$ deficiency caused by statin therapy (Draeger et al., 2006; Littarru & Langsjoen, 2007). Statins decrease plasma levels of $CoQ_{10}$ depending on the dose and statin class (Mabuchi et al., 2005; Stocker et al., 2006). In the present study, we used a less toxic hydrophilic statin in a moderate dose (pravastatin at 40 mg/kg bw) for the in vivo studies. We observed pancreatic islet dysfunction that was prevented by the antioxidant $CoQ_{10}$ diet supplementation. Pancreatic β cells are very sensitive to reactive oxygen species because they have limited antioxidant enzymes, which render them susceptible to death in pro-oxidant conditions (Lenzen, 2008; Lenzen, Drinkgern, & Tiedge, 1996). Noteworthy, pravastatin had no harmful effect on islets of treated wild-type mice and mild toxicity in INS1E cells. This finding suggests that the defects of cholesterol metabolism turn pancreatic islets more susceptible to the statin toxic effects. This might be related to the degree of islet oxidative stress, since we have previously shown that several tissues from these mice present higher mitochondrial derived reactive oxygen production as compared to wild-type mice (H. C. F. Oliveira et al., 2005).

Previous studies using ex vivo (rat islets) and in vitro (INS1E cells) approaches demonstrated that mevalonate or geranylgeranyl pyrophosphate supplementation could rescue impaired insulin secretion induced by lovastatin and simvastatin, respectively (Metz, Rabaglia, Stock, & Kowluru, 1993; Zúñiga-Hertz et al., 2015). Mevalonate reconstitutes the entire cholesterol biosynthesis pathway, including $CoQ_{10}$ synthesis, whereas geranylgeranyl pyrophosphate reconstitutes downstream steps that provide substrates for prenylation of numerous proteins. Isoprenylation of low molecular weight GTP-binding proteins is important for secretory granules trafficking in β cells (Metz et al., 1993; Sidarala, Veluthakal, Syeda, & Kowluru, 2015) and overall cell cycle progression and proliferation (Garcia-Ruiz, Morales, & Fernandez-Checa, 2012).

Statins cause cell death in various cell lines, mostly cancer and muscle cells (Cafforio, Dammacco, Gernone, & Silvestris, 2005; Costa, Fernandes, de Souza-Pinto, & Vercesi, 2013; Graaf, Richel, van Noorden, & Guchelaar, 2004; Guijarro et al., 1999; Werner, Sacher, & Hohenegger, 2004). These studies have implicated mechanisms involving oxidative stress and mitochondria dysfunction, such as inhibition of mitochondrial respiration and membrane potential disruption (Busanello et al., 2017; Costa et al., 2013; K. A. P. Oliveira, Zecchin, Alberici, Castilho, & Vercesi, 2008). Studies in primary healthy human skeletal muscle cells demonstrated that simvastatin and lovastatin induce apoptosis via the mitochondria pathway (Sacher, Weigl, Werner, Szegedi, & Hohenegger, 2005). Rat soleus muscle fiber biopsies incubated with simvastatin exhibited an inhibition of mitochondrial respiration and an increase in the rate of hydrogen peroxide production and reduction of $CoQ_{10}$ levels. Coincubation of muscle samples with $CoQ_{10}$ prevented the muscle toxic effects of simvastatin (La Guardia et al., 2013). *In vivo*, rats treated with atorvastatin exhibited muscle injury and decreased ATP content and insulin signaling. These effects were significantly improved with coadministration of $CoQ_{10}$ (El-Ganainy et al., 2016). Mice liver and muscle mitochondria from in vivo and in vitro treatment with lovastatin, respectively, presented higher susceptibility to mitochondrial membrane permeability transition, a process dependent on calcium and oxidative stress (Velho et al., 2006), that frequently precedes apoptotic or necrotic death (Figueira et al., 2013). Mice treated with atorvastatin for 2 months exhibited hepatic toxicity, increased oxidative damage and apoptosis (Pal, Ghosh, Ghosh, Bhattacharyya, & Sil, 2015). In addition, increased oxidative injury was verified in heterozygous familial hypercholesterolemic subjects treated with different statins for 6 months (Sinzinger, Chehne, & Lupattelli, 2002).

The above-mentioned studies of statin-induced toxicity focused on muscle, liver or tumor cells. Here, we focused on pancreatic islet because of the recent reported diabetogenic effect of statins. Our rational is that the endocrine pancreas is more susceptible to statin-induced oxidative stress given its poor antioxidant power and that islet from hypercholesterolemic subjects may be even more prone to statins induced oxidative stress. Recently, Urbano et al. (2017) showed that atorvastatin, but not pravastatin, impairs mitochondrial function in human pancreatic islets and INS1 β cells due to an oxidative stress. Like in our study, they showed that the atorvastatin effects were inhibited by an unspecific antioxidant, the N-acetylcysteine.

We demonstrated here that pravastatin increases $H_2O_2$ production in INS1 cells and $CoQ_{10}$ prevent this effect. To confirm that pravastatin induces oxidative stress in vivo in hypercholesterolemic mice islets, we showed an inhibition of aconitase activity, a sensitive target of superoxide and indicative of mitochondria redox state. This aconitase inhibition was prevented by $CoQ_{10}$ diet supplementation. These results are in agreement with recent data showing that pravastatin treated $LDLr^{-/-}$ mice had mitochondrial oxidative toxic effects in plantar muscle (Busanello et al., 2017) and liver (Marques et al., 2018) in association with reduced aconitase activity and increased lipoperoxidation. Thus, mitochondrial redox imbalance is probably the cause of islet toxic effect of chronic pravastatin treatment of hypercholesterolemic $LDLr^{-/-}$ mice.

Together, the present results demonstrate that in vivo, ex vivo, and in vitro treatments with statins impair β-cell redox balance, function (insulin secretion), and viability that are attenuated by $CoQ_{10}$. The proposed model is summarized in Figure 9. Pancreatic islets from hypercholesterolemic mice are more susceptible to statins toxic effects than islet from normolipidemic mice. Simvastatin and atorvastatin have more potent harmful effects on β-cell lineage than pravastatin. These data suggest that the detrimental effects of statins on the endocrine pancreas can be prevented by coadministration of $CoQ_{10}$, or other safe antioxidant.

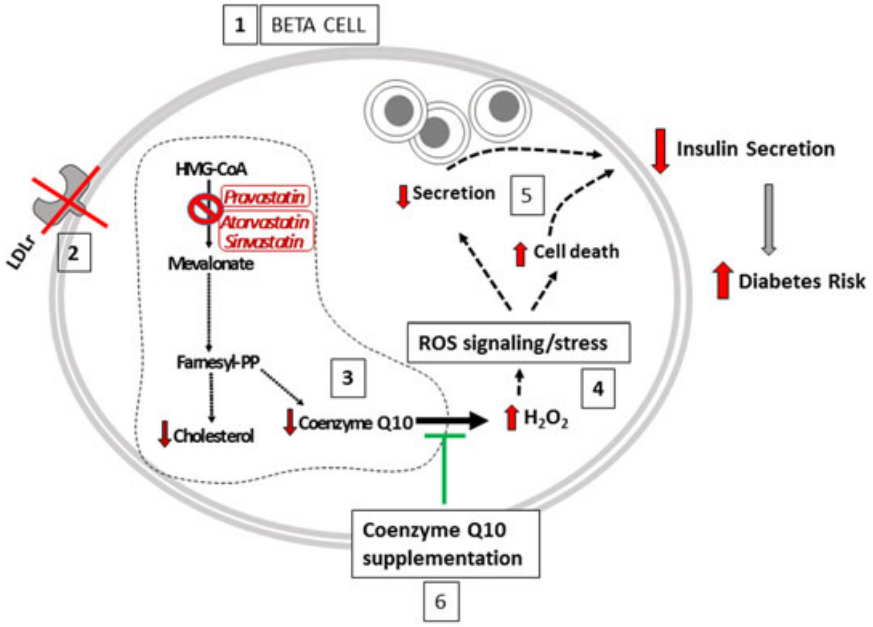


**FIGURE 9** Proposed model of how statins induce β-cell dysfunction and death in LDL receptor deficient cells. (1) β cells have low expression of antioxidant enzymes; (2) $LDLr^{-/-}$ tissues have low reducing power content (NADPH) due to high steroidogenesis rates; (3) statins decrease both steroidogenesis and the content of the antioxidant $CoQ_{10}$; (4) together, these conditions increase ROS; (5) ROS signaling and/or stress triggers downregulation of key secretory proteins and/or cell death leading to low insulin secretion and risk of diabetes development; (6) supplementation with $CoQ_{10}$ inhibits ROS signaling and/or stress and protects against cell toxicity. $CoQ_{10}$: coenzyme $Q_{10}$; LDL: low density lipoprotein; LDLr: LDL receptor ROS: reactive oxygen species [Color figure can be viewed at wileyonlinelibrary.com]

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## AUTHORS CONTRIBUTION

E. L. G., J. C. S., and H. C. F. O. conceived the study. E. L. G. and J. C. S. contributed equally. E. L. G., J. C. S., M. G. A., J. F. V., A. C. M., and A. G. S. conducted the experiments. J. R. T. conducted $CoQ_{10}$ liver and plasma measurements and performed the statistical analyses. E. L. G. and H. C. F. O. wrote the manuscript. All authors approved the final version of the manuscript.

## CONFLICTS OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## ORCID

*Helena C. F. Oliveira* http://orcid.org/0000-0003-0119-6992
*Jane C. de Souza* https://orcid.org/0000-0002-6687-2819

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