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Chronic use of pravastatin reduces insulin exocytosis and increases β-cell death in hypercholesterolemic mice

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ABSTRACT

We have previously demonstrated that hypercholesterolemic LDL receptor knockout ($LDLr^{-/-}$) mice secrete less insulin than wild-type mice. Removing cholesterol from isolated islets using methyl-beta-cyclodextrin reversed this defect. In this study, we hypothesized that in vivo treatment of $LDLr^{-/-}$ mice with the HMGCoA reductase inhibitor pravastatin would improve glucose-stimulated insulin secretion. Female $LDLr^{-/-}$ mice were treated with pravastatin (400 mg/L) for 1–3 months. Isolated pancreatic islets were assayed for insulin secretion rates, intracellular calcium oscillations, cholesterol levels, NAD(P)H and SNARE protein levels, apoptosis indicators and lipidomic profile. Two months pravastatin treatment reduced cholesterol levels in plasma, liver and islets by 35%, 25% and 50%, respectively. Contrary to our hypothesis, pravastatin treatment increased fasting and fed plasma levels of glucose and decreased markedly (40%) fed plasma levels of insulin. In addition, ex vivo glucose stimulated insulin secretion was significantly reduced after two and three months (36–48%, $p < 0.05$) of pravastatin treatment. Although reducing insulin secretion and insulinemia, two months pravastatin treatment did not affect glucose tolerance because it improved global insulin sensitivity. Pravastatin induced islet dysfunction was associated with marked reductions of exocytosis-related SNARE proteins (SNAP25, Syntaxin 1A, VAMP2) and increased apoptosis markers (Bax/Bcl2 protein ratio, cleaved caspase-3 and lower NAD(P)H production rates) observed in pancreatic islets from treated mice. In addition, several oxidized phospholipids, tri- and diacylglycerols and the proapoptotic lipid molecule ceramide were identified as markers of pravastatin-treated islets. Cell death and oxidative stress (H_2O_2 production) were confirmed in insulin secreting INS-1E cells treated with pravastatin. These results indicate that chronic treatment with pravastatin impairs the insulin exocytosis machinery and increases β-cell death. These findings suggest that prolonged use of statins may have a diabetogenic effect.

1. Introduction

Type 2 diabetes mellitus (T2D) is related to both insulin resistance in target tissues and pancreatic islet malfunction and failure (Halban et al., 2014). Progression from the insulin-resistant state to T2D occurs in a subset of individuals in whom β-cells are unable to maintain increased insulin secretion upon a chronic demand. In these cases, β-cells become exhausted and dysfunctional with reduced secretory response to glucose stimulus, reduced insulin synthesis and eventually β-cell apoptosis. The reasons why β-cells become dysfunctional in some individuals whereas others remain in an insulin resistant state for prolonged periods are not well understood. Several hypotheses including glucotoxicity (Gleason et al., 2000) and lipotoxicity (Unger, 1995) caused by chronic hyperglycemia and chronic hyperlipidemia, respectively, have been raised. It has been suggested that the lipotoxicity associated with increased free fatty acid accumulation in β-cells, as occurs in obesity, can cause β-cell death (Poitout and Robertson, 2008).

Previous in vitro studies have indicated that cholesterol may also play a major role in controlling β cell function (Vikman et al., 2009). In vivo studies also demonstrated that cholesterol accumulation in β-cells is associated with reduced insulin secretion, β-cell dysfunction and decreased β-cell mass in genetic

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modified mouse models such as the β-cell-specific ABCA1 knockout and SREBP-2 transgenic (Brunham et al., 2007; Fryirs et al., 2009; Hao et al., 2007; Ishikawa et al., 2008). In pancreatic β-cell membranes, cholesterol and sphingolipids form lipid raft domains that are important to anchor membrane proteins involved in the secretory process such as the voltage-gated calcium channel $Ca_v2.1$ (Chamberlain et al., 2001; Taverna et al., 2004) and the SNARE proteins (soluble *N*-ethylmaleimide sensitive factor attachment receptor) (Taverna et al., 2004; Wiser et al., 1999) that drive insulin vesicle fusion with the plasma membrane (Bruns and Jahn, 2002; Hanson et al., 1997).

We previously demonstrated that hypercholesterolemic LDL receptor knockout mice ($LDLr^{-/-}$) exhibited impaired insulin secretion due to lower glucose uptake and metabolism, reduced PKAα and SNARE proteins expression and calcium handling deficiency. These disturbances were reversed by removing cholesterol from isolated islets in vitro with methyl beta cyclodextrin. (Bonfleur et al., 2010, 2011; Souza et al., 2013). Therefore, we proposed that primary (genetic) hypercholesterolemia increases the risk of diabetes development. Here, we hypothesized that treating these hypercholesterolemic mice with inhibitors of the 3-hydroxy-3-methyl-glutaryl coenzyme A reductase (HMGCoA red) would decrease islet cholesterol content and recover the insulin secretory capacity of $LDLr^{-/-}$ mice. Statins have been used to treat hypercholesterolemic patients for cardiovascular disease prevention since the late 80s (Taylor et al., 2013). These drugs are first-line agents that effectively reduce LDL-cholesterol. Other beneficial pleiotropic effects of statins have been reported such as improvements in endothelial function and stabilization of atherosclerotic plaques as well as anti-inflammatory and antioxidant actions (Davignon et al., 2004). However, adverse effects have also been identified since statins have been available, and two of the most common clinician concerns are myopathy and diabetes (Desai et al., 2014).

2. Materials and methods

2.1. Animals

Low-density lipoprotein receptor knockout ($LDLr^{-/-}$) 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 experiments were approved by the University's Committee for Ethics in Animal Experimentation (CEUA/UNICAMP, protocol # 3001–1). 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 h light/dark cycle. Female mice (4 weeks old) were treated with pravastatin for 4–12 weeks dissolved in the drinking water (400 mg/L). The estimated pravastatin sodium (Medley) dose of 40 mg/kg body weight per day was based on the drink consumption rate measurements (3.6 mL/day). Controls received filtered tap water without pravastatin. Additional groups of wild type mice (C57BL6/J) were also treated with pravastatin.

2.2. Plasma biochemical analyses

Blood glucose was measured using a glucose analyzer (Accu-Chek Advantage; Roche Diagnostics, Basel, Switzerland), and plasma cholesterol, triglycerides and free fatty acids were measured using standard commercial kits (Roche Diagnostics GMbH, Mannheim, Germany; Wako Chemical, Neuss, Germany) according to the manufacturer's instructions. Blood samples were obtained between 8 and 9:00 am after a 12 h fasting period (food was removed at 8:00 pm).

2.3. Oral glucose tolerance test (OGTT) and insulin tolerance test (ITT)

After 12 h of fasting (food removed at 8:00 pm and blood obtained between 8 and 9:00 am), mice received an oral dose of glucose solution (1.5 g/kg body weight). Basal blood samples were collected from the tail tip before (t = 0 min) and 15, 30, 60, 90 and 120 min after glucose ingestion. For the ITT, blood was taken from mice that had been fasted for 3 h before (t = 0 min) and 5, 10, 15, 30 and 60 min after an i.p. insulin injection (0.75 U/kg body weight, regular human insulin, Eli Lilly Co.) for glucose analysis.

2.4. 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 were then selected with a microscope (Boschero and Delattre, 1985). Four replicates of four islets/well in each condition (basal and glucose stimulated) from each mouse (n = 4–8 mice per group) were used for the insulin secretion assay. Islets were pre-incubated 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 HEPES, supplemented with 5.6 mmol/L glucose and 0.3% BSA, equilibrated with a mixture of 95% O_2/5% CO_2, pH 7.4. The islets were further incubated during 1 h in KBB containing glucose (2.8 or 11.1 mmol/L). At the end of the incubation period, media insulin content was measured by radioimmunoassay (Scott et al., 1981).

2.5. Cytoplasmatic Ca^{+2} and NADPH response to glucose

For intracellular calcium recordings, islets were incubated with fura-2 acetoxymethyl ester (5 μM) for 1 h at 37 °C in KBB buffer that contained 5.6 mM glucose, 0.3% BSA and pH 7.4. Ca^{+2} recordings in whole islets (8 fields per islet, six islets per mouse, 7 mice per group) were obtained by imaging intracellular Ca^{+2} using an inverted epifluorescence microscope (Nikon UK, Kingston, UK), a digital camera (Hamamatsu Photonics, Barcelona, Spain) and a dual-filter wheel (Sutter Instrument Co., Navato, CA) that was equipped with 340 and 380 nm filters, 10 nm band-pass, as previously described (Rafacho et al., 2010). Fluorescence recordings were expressed as the ratio of fluorescence at 340 and 380 nm (F340/F380). NAD(P)H auto-fluorescence was monitored using the imaging system described above. NAD(P)H auto-fluorescence was excited with a 365 nm filter, whereas emission was filtered at 445 ± 25 nm, as previously described (Rafacho et al., 2010). Six fields per islet, 6 islets/mouse, 5 mice per group.

2.6. Western blotting

Islets (150–200 per mouse) were homogenized in urea lysis buffer and treated with Laemmli loading buffer containing dithiothreitol. After heating to 95 °C for 5 min, the proteins were separated by electrophoresis (40 μg protein/lane, 12% or 10% gels) and were then transferred to nitrocellulose membranes. The nitrocellulose membranes were treated for 1.5 h with a blocking buffer (5% BSA, 10 mmol/L tris, 150 mmol/L NaCl, and 0.02% Tween 20) and were subsequently incubated with the following primary antibodies: Caspase-3 (Millipore), BAX (Cell signaling), Bcl-2 (Cell signaling), SNAP25 (Sigma), VAMP-2 (Calbiochem), Syntaxin-1A (Santa Cruz Biotechnology), IDE (Abcam), HMGCoA red (Millipore). Rabbit polyclonal antibody against GAPDH (Santa Cruz Biotechnology) was used as an internal control. Membranes were then incubated with anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibodies (1:10000, Invitrogen). Detection was performed using enhanced chemiluminescence (SuperSignal