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Research Paper

Dual Effect of Rosuvastatin on Glucose Homeostasis Through Improved Insulin Sensitivity and Reduced Insulin Secretion

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ABSTRACT

Statins are beneficial in the treatment of cardiovascular disease (CVD), but these lipid-lowering drugs are associated with increased incidence of new on-set diabetes. The cellular mechanisms behind the development of diabetes by statins are elusive. Here we have treated mice on normal diet (ND) and high fat diet (HFD) with rosuvastatin. Under ND rosuvastatin lowered blood glucose through improved insulin sensitivity and increased glucose uptake in adipose tissue. *In vitro* rosuvastatin reduced insulin secretion and insulin content in islets. In the beta cell Ca^{2+} signaling was impaired and the density of granules at the plasma membrane was increased by rosuvastatin treatment. HFD mice developed insulin resistance and increased insulin secretion prior to administration of rosuvastatin. Treatment with rosuvastatin decreased the compensatory insulin secretion and increased glucose uptake. In conclusion, our data shows dual effects on glucose homeostasis by rosuvastatin where insulin sensitivity is improved, but beta cell function is impaired.

1. Introduction

Western life style with excessive food intake and reduced physical activity is the leading cause of metabolic syndrome, including the following criteria; increased abdominal waist line, elevated triglycerides, low HDL cholesterol levels, high blood pressure and insulin resistance (Grundy et al., 2004). Metabolic syndrome is a strong predictor for the development of cardiovascular disease (CVD), the leading cause of mortality world-wide (Preiss and Sattar, 2012). The major risk factor for CVD is hypercholesterolemia, and therefore statins are the major therapeutic drugs used to prevent cardiovascular episodes. Statins decrease levels of low density lipoprotein cholesterol (LDL) in the blood by inhibiting 3-hydroxy-3-methyl-glutaryl coenzyme-A (HMG-CoA). Although proven beneficial for the treatment of CVD, there is emerging evidence suggesting increased incidence of new-onset diabetes with statin use (Cederberg et al., 2015; Mora et al., 2010; Preiss and Sattar, 2012; Ridker et al., 2008; Ruscica et al., 2014; Sattar et al., 2010). The first study to report an increased incidence of diabetes with statins was the JUPITER trial, a double-blind randomized study comparing subjects assigned to rosuvastatin 20 mg or placebo (Mora et al., 2010; Ridker et al., 2008). Rosuvastatin has hydrophilic properties and is more potent in reducing cholesterol levels than pravastatin and simvastatin (Paoletti et al., 2001).

The mechanisms behind increased diabetes incidence by statins remain to be investigated. A follow-up study in the METSIM cohort showed association between increased risk of diabetes with statins and impaired insulin sensitivity and insulin secretion (Cederberg et al., 2015). Others suggest improved insulin sensitivity by statins (Guclu et al., 2004; Okada et al., 2005; Paolisso et al., 1991; Sonmez et al., 2003). Insulin resistance leads to an increased pressure on the beta cells to secrete more insulin. When the beta cell response is insufficient, fasting and postprandial hyperglycemia develops ultimately leading to type 2 diabetes. Normally, increased blood glucose stimulates beta cells to secrete insulin through a Ca^{2+} dependent process. We have previously demonstrated that cholesterol in the plasma membrane of the beta

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cell is essential for insulin secretion, and removal reduces glucose-stimulated insulin secretion by >50% (Vikman et al., 2009). Thus, although elevated blood cholesterol is deleterious, sufficient cholesterol levels in cell membranes are vital. Hence, the balance between circulating and cellular levels needs to be tightly controlled. It is not known whether statins influence glucose metabolism through this balance or through a direct effect on beta cells. In favor of the latter, studies performed in cell lines suggest effects on small G-binding proteins (Li et al., 1993) and reduced voltage-dependent Ca^{2+} influx (Okada et al., 2005; Salunkhe et al., 2016; Yada et al., 1999). Moreover, acute experiments with rosuvastatin *in vitro* in human islets have shown ultrastructural changes (Bugliani et al., 2013) and reduced insulin secretion (Zhao and Zhao, 2015).

The High Fat Diet (HFD) fed mouse is a widely used model to study insulin resistance (Winzell and Ahren, 2004), and the phenotype has many of the risk factors associated with metabolic syndrome (Grundy et al., 2004). The mice are obese, and have elevated cholesterol and blood glucose. The blood glucose is only slightly increased since the HFD mouse compensate with increased insulin secretion, further fueling weight gain and insulin resistance (Winzell and Ahren, 2004). As many patients treated with statins have a high BMI, it is of interest to investigate the mechanisms explaining the effect of statin treatment on glucose homeostasis in HFD mice compared to mice fed a normal diet (ND). Here we have studied the integrated role of rosuvastatin on glucose homeostasis and aimed to understand the cellular mechanisms by which rosuvastatin acts on insulin secretion and glucose uptake.

2. Materials and Methods

2.1. Animals

C57BL/6 female mice were delivered from Taconic, Denmark at 8 weeks. Mice were allowed to adapt to the surrounding for one week before given high-fat diet (HFD) and matched low-fat normal diet (ND) (Research Diets Inc, New Brunswick, USA). The ND contained the same ingredients with a closely matched composition to the HFD formula. After 4 weeks on ND or HFD mice part of the mice were given rosuvastatin (0.2 mg/mice/day; LKT Laboratories, MN, USA) in the drinking water for the rest of the study period. At the end of the study mice were sacrificed and tissues were collected for further analysis. The procedures used in the *in vitro* and *in vivo* studies were approved by the ethical committee of Lund/Malmö.

2.2. OGTT and In Vivo Measurements

The *in vivo* study was performed according to the study plan (Fig. S1). OGTT was performed at week 0, 4, 8 and 12. Prior to OGTT mice were fasted for 4–6 h. D-Glucose was administered at 75 mg/mouse by oral gavage and blood was sampled from the vena saphena at 0, 15, 30, 60 and 120 min. The samples were immediately analyzed for glucose using a blood glucose meter (Accu-Check Aviva, Roche Diagnostics Scandinavia AB, Bromma, Sweden). Plasma samples were stored at −20 °C until being assayed for insulin ELISA (Mercordia, Uppsala, Sweden). Blood samples to measure total cholesterol, triglycerides and HDL were collected at week 6, 10 and 12 and levels were determined using commercially available kits (HDL: HDL-cholesterol plus direct method (#981823); total cholesterol: InfinityTM Cholesterol Liquid Stable Reagent (#TR13421); triglycerides: InfinityTM Triglycerides Liquid Stable Reagent (#TR22421); Thermo Fisher Scientific, VA, USA). LDL was estimated according to the manufacturer's instructions.

2.3. Isolation of Islets and Hormone Release Assays

Pancreatic islets were isolated by collagenase digestion and hand-picked prior to insulin secretion measurements. Insulin secretion was measured in static batch incubations as previously described (Vikman et al., 2009). Briefly, islets were pre-incubated in 1 mM glucose for 30 min followed by 1 h incubation in Krebs-Ringer bicarbonate buffer (pH = 7.4) with 10 mM HEPES, 0.1% bovine serum albumin and variable glucose as indicated (5.6, 11.1, and 16.7 mM). In some experiments the buffer was supplemented with 0.1 μM GLP-1 (Bachem, Bubendorf, Switzerland) or 50 mM KCl (equimolar substituted for NaCl), respectively. Total insulin islet content was determined after extraction with acidic ethanol. Insulin secretion and islet content was measured using Radio Immuno Assay (RIA; Millipore, Solna, Sweden).

2.4. Ultrastructural Analysis

Mouse islets were fixed in 2.5% glutaraldehyde in freshly prepared Millonig's buffer and post-fixed in 1% osmium tetroxide before being dehydrated, embedded in AGAR 100 (Oxford Instruments Nordiska AB, Johanneshov, Sweden) and cut into ultrathin sections (70–90 nm). The sections were put on Cu-grids and contrasted using uranyl acetate and lead citrate. The islet containing sections were examined in a JEM 1230 electron microscope (JEOL-USA, Inc., Peabody, USA). Micrographs were analyzed with respect to the intracellular granule distribution as described elsewhere (Vikman et al., 2009).

2.5. Imaging and Ca^{2+} Measurements

Imaging of insulin and glucagon staining was performed using confocal microscopy imaging (Zeiss LSM 510). For calcium imaging islets were loaded with 4 μM Fura 2-AM (TefLabs, Austin, USA) for 40 min followed by 30 minutes de-esterification in imaging buffer at pH 7.4 (mM: KCl 3.6, $MgSO_4$ 0.5, $CaCl_2$ 2.5, NaCl 140, $NaHCO_3$, NaH_2PO_3 0.5, HEPES 5). Imaging was performed with a Polychrome V monochromator (TILL Photonics, Graefelfing, Germany) on a Nikon Eclipse Ti Microscope (Nikon, Tokyo, Japan) equipped with an ER-BOB-100 trigger, an iXON3 camera, and iQ2 software (Andor Technology, Belfast, UK). Recordings were performed at one frame per second at 37 °C under perfusion at 1 ml/min. A region was marked around each islet and the ratio of fluorescence emission intensity per unit area (μm^2) at 340 nm (exposure 150 ms) and 380 nm (exposure 100 ms) was recorded. Parameters analyzed are described in Suppl Table 1.

2.6. Isolation and Glucose Uptake in Primary Adipocytes

Primary adipose cells were isolated from epididymal adipose tissue as previously described (Rodbell, 1964). The isolated cells were suspended (5% suspension) in Krebs-Ringer (KRH) medium containing 25 mM HEPES pH 7.4, 200 nM adenosine, 2 mM glucose and 3% BSA (w/v), and glucose uptake measured as previously described (Gliemann et al., 1984). Briefly, cells were incubated in KRH medium (37 °C, shaking water bath) in triplicates with or without insulin (28 nM) for 30 min, followed by addition of D-^{14}C(U)-glucose (2.5 μl/ml, NEC042, Perkin Elmer), and incubated for 30 min. The uptake was terminated by spinning 300 μl of each cell suspension in microtubes containing 80 μl dinonylphtalate oil. The cell fraction was collected, dissolved in scintillation fluid (Optima Gold, Perkin Elmer, Upplands Väsby, Sweden) and subjected to scintillation counting.

2.7. mRNA Expression Analysis

Pancreatic islets were lysed in Qiazol (Qiagen, Sollentuna, Sweden) and homogenized by vortexing. Total RNA was extracted using miRNeasy®Mini Kit protocol (Qiagen) and RNA concentration was measured on a NanoDrop (Thermo Scientific, Göteborg, Sweden). High Capacity cDNA Reverse Transcriptase Kit (Life Technologies, Stockholm, Sweden) was used to generate RT-PCR according to the manufacturer's protocol.

The QuantStudio™ 7 Flex Real-Time PCR System (Life Technologies) was used to performed qPCR according to the TaqMan®Universal PCR Master Mix I protocol (Life Technologies) using the following primers