Relazione Borsa di Studio FO.DI.RI.-MSD 2014/2015

Proponent: Francesca Urbano, Ph.D. Catania

Project TITLE: **Statins and risk of diabetes: A molecular study on new relevant issues.**

INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of mortality worldwide, causing approximately 17.3 million deaths in 2008 [1]. Hypercholesterolemia is one of the major CVD risk factors, and therefore is a very important therapeutic target [2]. Statins (3-hydroxy-methylglutaryl coenzyme A reductase inhibitors) are increasingly and widely prescribed cholesterol-lowering drugs for the prevention of cardiovascular disease (CVD) [3]. They have documented benefits in both the primary and secondary prevention of atherosclerotic cardiovascular disease (CVD) events [4, 5]. Statins mainly act to decrease low-density lipoprotein-cholesterol (LDL-C). Yet pleiotropic effects have been identified, such as improvements of endothelial function, stabilization of atherosclerotic plaques, and anti-inflammatory actions [6]. However, as the prescription rates for statins have increased, more adverse effects have been identified, with the most common being increased liver enzymes and myopathy [7].

Recently, there has been much investigation into the potential unforeseen adverse effects of statins, specifically the development of type 2 diabetes mellitus (T2DM). In February 2012, the Food and Drug Administration has added an adverse event warning to statin labels, stating that statins have been associated with increased glycosylated hemoglobin (A1C) and fasting blood glucose levels [FDA drug safety communication: important safety label changes to cholesterol-lowering statin drugs http://www.fda.gov/Drugs/DrugSafety/ucm293101.htm Accessed February 28, 2012]. Trials and meta-analyses have reported conflicting results regarding new-onset diabetes with statins.

The first report of the statin and diabetes link was in the original Justification for the Use of Statins in Primary Prevention: An Intervention Trial Evaluating rosuvastatin (JUPITER) trial. This revealed that subjects randomized to rosuvastatin 20 mg daily over the 1.9-year trial developed a significantly higher incidence of physician-reported type 2 diabetes [8]. A post hoc analysis of the JUPITER trial concluded that the positive cardiovascular benefits of statin-treated subjects outweighed the risk of developing diabetes [9]. It was the first study to report an increased incidence of diabetes with statins [8]. However, rather than an increase in new-onset diabetes, the West of Scotland Coronary Prevention Study (WOSCOPS), have suggested a protective effect of

pravastatin in preventing the development of diabetes [10]. Multiple other clinical studies have implicated atorvastatin, rosuvastatin, and simvastatin with inducing increased fasting blood glucose levels and increased hemoglobin A1c (HbA1c) [11-23]. Moreover, clinical trial evidence suggests that statin-induced diabetes appears to be a class effect (lipophilic or hydrophilic) and directly related to the intensity of therapy and degree of attained LDL cholesterol lowering [24]. Lipophilic statin penetrate the cell membrane easier those which are hydrophilic, and therefore are likely to have more extrahepatic effects. However, although meta-analyses and reviews of statins have shown that there is an increased risk of new-onset diabetes, statins' ability to decrease major cardiovascular events and mortality outweighs this risk, and no change in clinical practice is currently recommended [25, 26].

The molecular mechanisms that underlies statins leading to an accelerated progression to diabetes remains unclear. Evidence exists for changes in Ca2+ channel function in pancreatic beta cells, reduced translocation of glucose transporter 4 (GLUT4) in insulin target tissues, decreased adipocyte differentiation, reduction of other important downstream products such as coenzyme (Q10), isoprenoid and dolichol; their depletion leads to reduced intracellular signaling. Other possible mechanisms implicated in the effect of statins on new-onset diabetes are: statin interference with intracellular insulin signal transduction pathways via inhibition of necessary phosphorylation events and reduction of small GTPase action; inhibition of adipocyte differentiation leading to decreased peroxisome proliferator activated receptor gamma and CCAAT/enhancer-binding protein which are important pathways for glucose homeostasis; decreased leptin causing inhibition of β-cells proliferation and insulin secretion; diminished adiponectin levels; as well as new avenues, such as UCP3 changes and miRNA deregulation [27].

Most likely some combination of these effects contributes to the diabetogenic effects of statins observed in several meta-analyses and clinical trials.

However, molecular mechanisms are often briefly discussed, and currently they remain unclear, it is then necessary examine them in depth. Some topics of particular interest have not yet been addressed, it is still unknown, for example, if the statin treatment could affect incretin effect and GLP-1 secretion, there is no data concerning the specific effect of the insulin resistance caused by the drug on alfa cell and glucagon secretion and there are still not many information about microRNAs involved in this biological process.

OBJECTIVE

In this project we tried to elucidate the cellular and molecular mechanisms underlying the development of diabetes in association with statin use.

In particular we have studied the effect of a chronic treatment with atorvastatin or pravastatin (only in specific sets of experiments) at different concentrations (10, 100 ng/ml) and for different periods of time (24, 48 hours) on: insulin secretion in β-cells, GLP-1 secretion in L-cells and insulin sensitivity in pancreatic α-cells.

MATERIALS AND METHODS

Cell models

We used different cell models: murine α-TC1-6 (pancreatic α-cell line) obtained from American Type Culture Collection (ATCC); rat INS-1 cell line (pancreatic β-cells model), a kind gift from C. B. Wollheim, University of Geneva, Geneva, Switzerland and murine GLUTag L-cells, kindly provided by Prof. Gribble, University of Cambridge, UK.

Chemicals and reagents

Cell media, antibodies and all chemicals were obtained from: Sigma Chemical, St. Louis, MO;. Santa Cruz, CA; UBI, Lake Placid, NY; Millipore, Billerica, MA; Cell Signaling Technology, Inc. Boston, MA; R&D Systems, Minneapolis, MN; Alpco Immunoassays, Salem, NH; Pierce Chemical, Dallas, TX.

Chronic exposure to atorvastatin or pravastatin

Twenty-four hours after planting, the cells (α-TC1-6, INS-1, GLUTag) were cultured for 24 or 48 hours at 37°C in complete medium in the presence or absence of atorvastatin or pravastatin (10 or 100 ng/ml). Medium was replaced every 24 hours.

Insulin secretion in INS-1 cells

After treatments, on the day of the experiment, INS-1 cells were washed twice with glucose-free Krebs-Ringer medium (KRB), pre-incubated for 1 hour in KRB with 2.8 mmol/l glucose and than re-incubated for 1 hour at 37 °C in the presence of different glucose concentrations (2.8, or 22.2 mmol/l). At the end of the incubation period, the media were collected and centrifuged to remove any floating cells. Insulin was measured in the supernatants using an enzyme-linked immunosorbent assay (Millipore Co.).

Measurement of ATP content in INS-1 cells

The ATP levels were measured using the CellTiter-Glo Luminescent Cell Viability Assay

(Promega, Madison, WI, USA) according to the manufacturer's protocol. Briefly, equal numbers of cells were cultured for 24 or 48 hours at 37°C in complete medium in the presence or absence of atorvastatin or pravastatin (10 or 100 ng/ml). Then, the cells were washed and acutely exposed to different glucose concentrations (0, or 22.2 mmol/l). The luminescent signal was proportional to the amount of ATP.

GLP-1 secretion in GLUTag cells

After treatment with atorvastatin, on the day of the experiment, GLUTag cells were washed twice with glucose-free Krebs-Ringer buffer (KRB) and than incubated in KRB for 2 h at 37 °C in the presence of different glucose concentrations (0 or 5 mmol/l). At the end of the incubation period, the media were collected and centrifuged to remove any floating cells. GLP-1 levels in the supernatants were determined using an enzyme-linked immunosorbent assay (ALPCO).

Insulin stimulation in α-TC1-6 and GLUTag cells

To assess the effect of insulin, after treatment with atorvastatin the cells were serum-starved for 2 hours in medium with BSA 1% instead of FBS before stimulation with insulin. Acute stimulation with 10^{-9} M insulin was performed for five minutes.

Western blot analysis

Cellular proteins were extracted using a radio-immunoprecipitation assay (RIPA) lysis buffer. The protein concentrations were determined by the BCA assay and the cell extracts were stored at -20°C. Equal amounts of protein were separated by SDS-PAGE.

All of the immunoblot signals were visualized using the ECL method, auto-radiographed and subjected to densitometric analyses with the ImageJ ™ software version 1.41.

Glucagon secretion

α-TC1-6 cells were cultured for 24 or 48 hours at 37°C in complete medium in the presence or absence of atorvastatin (10 or 100 ng/ml). At the end of this period cells were serum starved for two hours and incubated for 2 h in Krebs-Ringer buffer (KRB) containing 16.7 mmol/liter glucose and 0.5% BSA (pH 7.4) in the presence or absence of atorvastatin and/or insulin (10^{-9} M).

Samples were collected in vials containing aprotinin (0.1 mg/ml) and kept frozen at −20°C for glucagon ELISA analysis (Mercodia AB).

Statistics

All data will be analyzed with an unpaired two-tailed Student's t test or analysis of variance (ANOVA) and post hoc tests as appropriate. A p value less than 0.05 will be considered statistically significant.

RESULTS

Insulin secretion in INS-1 cells after atorvastatin or pravastatin treatment

To study the effects of a chronic treatment with atorvastatin on insulin release, we investigated acute glucose-stimulated insulin secretion in control cells and in cells that had been chronically pre-exposed to atorvastatin (10 or 100 ng/ml for 24 or 48h).

In the control group 22.2mmol/l glucose acutely stimulated insulin secretion.

Pre-incubation with atorvastatin impaired both basal and glucose-stimulated insulin release in a time and dose-dependent manner with a maximal effect observed after 48 h exposure to atorvastatin 100 ng/mL (Fig. 1). In contrast, in INS-1 cells that had been pre-exposed to pravastatin both basal and glucose-induced insulin secretion were unaffected for all of the tested dose-time combinations (Fig. 2).

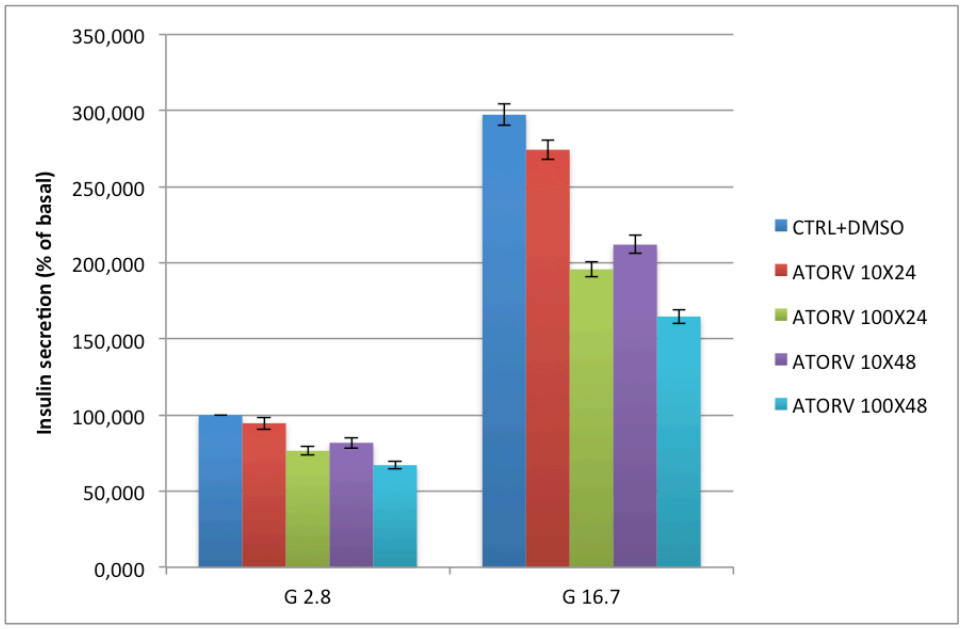

Figure 1: ***Effect of atorvastatin (10 or 100 ng/ml for 24 or 48h) on glucose-induced insulin release in INS-1 cells.***

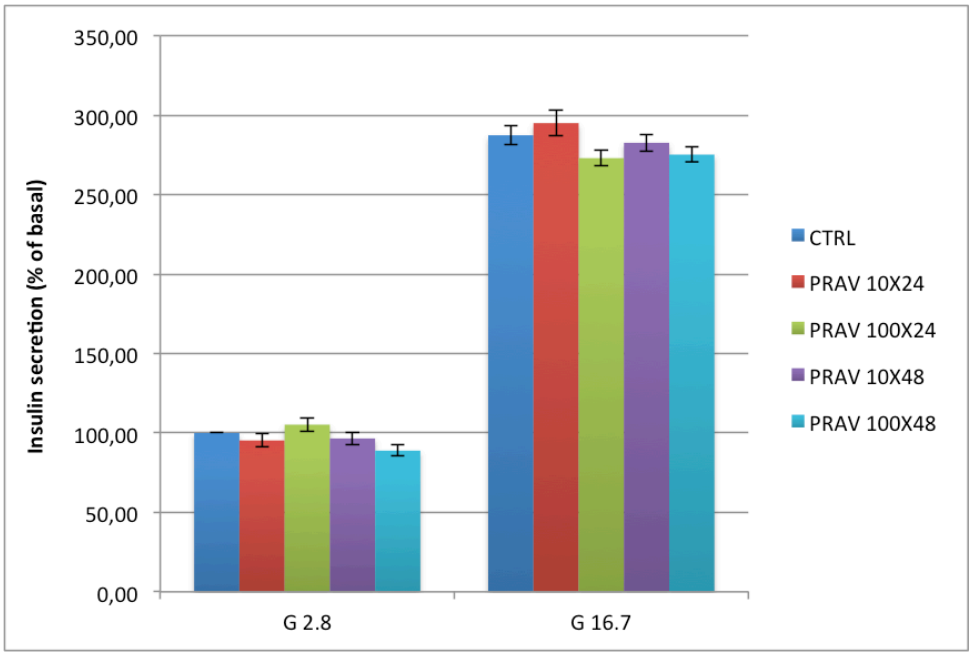

Figure 2: ***Effect of pravastatin (10 or 100 ng/ml for 24 or 48h) on glucose-induced insulin release in INS-1 cells.***

ATP production in INS-1 cells after atorvastatin or pravastatin treatment

Since insulin secretion is triggered by elevations in cytosolic ATP subsequent to metabolic fluxes, we evaluated ATP production in control cells and in cells that had been pre-exposed to atorvastatin or pravastatin (10 or 100 ng/ml for 24 or 48h).

In the control group, 22.2 mmol/l glucose acutely stimulated ATP production in compared with ATP levels observed in the absence of acute glucose stimulation.

In cells that had been pre-exposed to atorvastatin ATP production was markedly reduced (Fig. 3). In contrast, in INS-1 cells that had been pre-exposed to pravastatin both basal and glucose-induced ATP production were unaffected by treatment (Fig. 4).

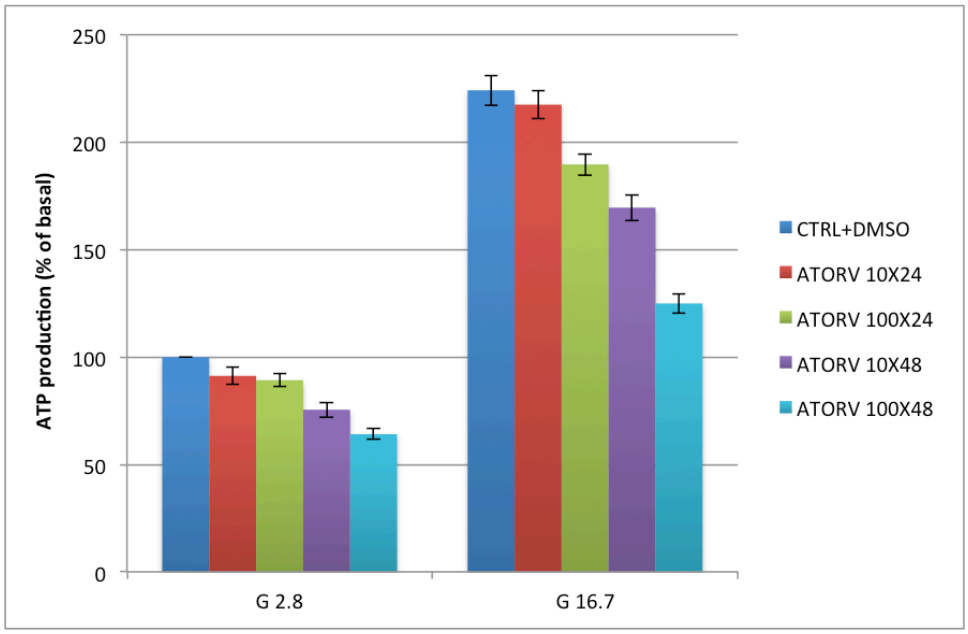

Figure 3: ***Effect of atorvastatin (10 or 100 ng/ml for 24 or 48h) on glucose-induced ATP production in INS-1 cells.***

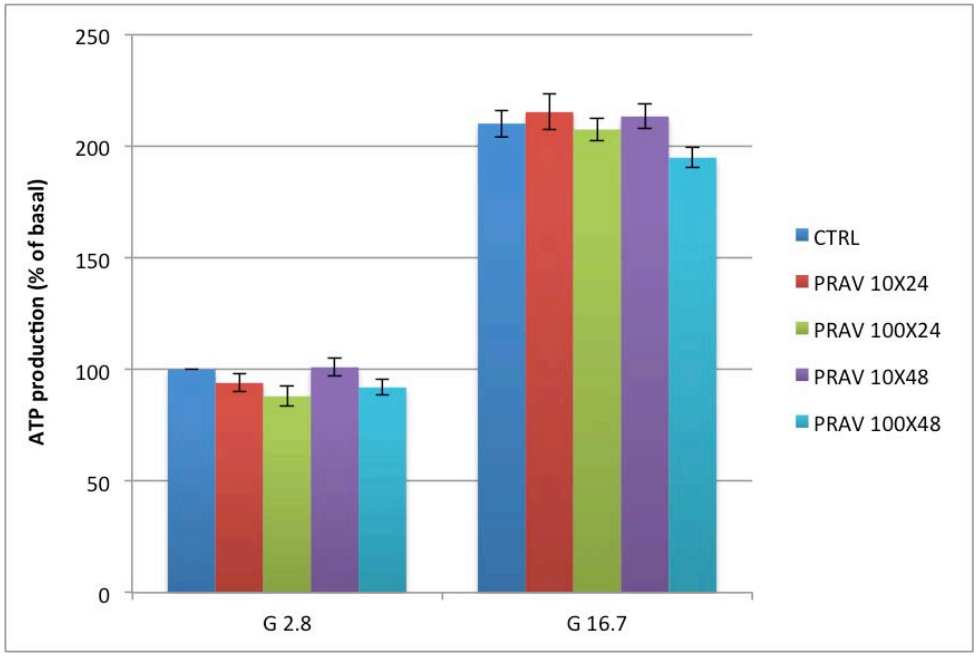

Figure 4: ***Effect of pravastatin (10 or 100 ng/ml for 24 or 48h) on glucose-induced ATP production in INS-1 cells.***

<u>GLP-1 secretion in GLUTag cells after atorvastatin treatment</u>

Since GLP-1 is known to elicit a potentiation of glucose-stimulated insulin secretion we evaluated the effects of a chronic treatment with atorvastatin on GLP-1 release. We investigated acute glucose-stimulated GLP-1 secretion in control cells and in cells that had been pre-exposed to atorvastatin (10 or 100 ng/ml for 24 or 48h).

While in the control group, 5 mmol/l glucose acutely stimulated GLP-1 secretion compared with its secretion in the absence of acute glucose stimulation, in cells that had been pre-exposed to atorvastatin glucose-stimulated GLP-1 release was markedly reduced (Fig. 5).

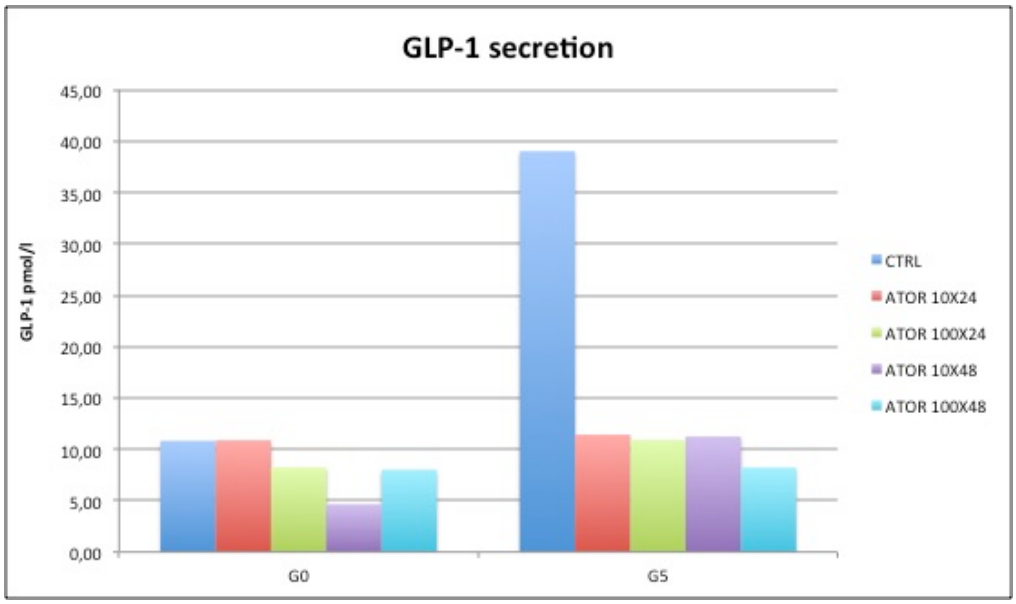

Figure 5: ***Effect of atorvastatin (10 or 100 ng/ml for 24 or 48h) on glucose-induced GLP-1 release in GLUTag cells.***

Insulin sensitivity in pancreatic α-cells after atorvastatin treatment

- IRS-1 Phosphorylation

Since statins were shown to induce insulin resistance in different cell types [24, 25] and insulin sensitivity is correlated with glucagon secretion in alpha cells [28], we investigated the effects of a chronic treatment with atorvastatin on insulin signaling in a model of pancreatic alpha cells, αTC1-C6. We evaluated the IRS-1-P Tyr612 protein expression, the proximal substrate of the intracellular IR (Insulin Receptor) pathway. Our data showed that insulin 10^{-9} M stimulated IRS-1-P (Tyr612) in control cells while in atorvastatin pre-exposed cells, the insulin effect on IRS-1-P was greatly reduced as evidenced by Western blot (Fig. 6).

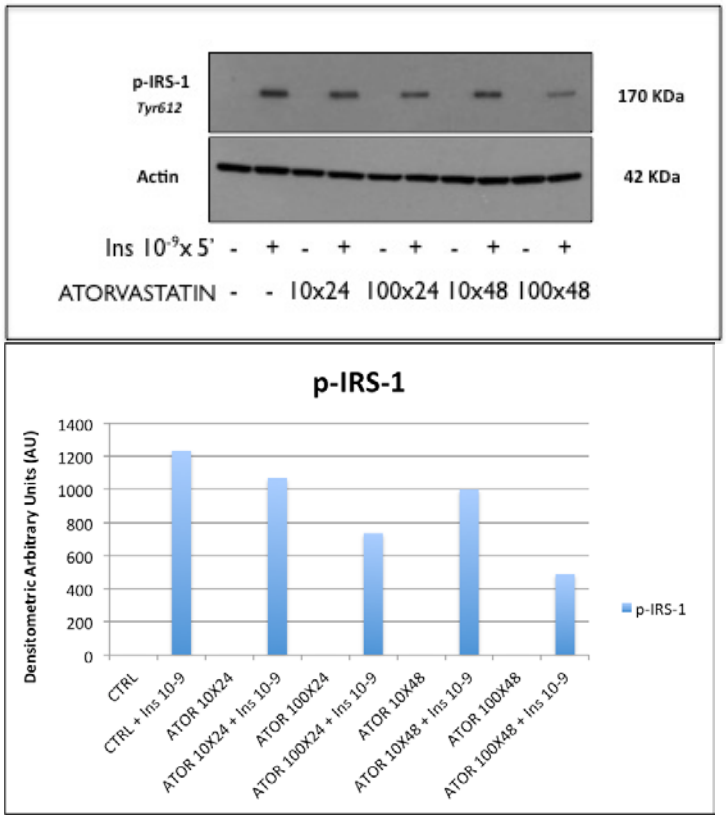

Figure 6: ***Effect of atorvavastatin (10 or 100 ng/ml for 24 or 48h) on IRS-1 phosphorylation (Tyr612) in αTC1-C6 cells.***

- Glucagon secretion

We investigated acute insulin-inhibition on glucagon secretion in control cells and in cells that had been pre-exposed to atorvastatin (10 or 100 ng/ml for 24 or 48h). After the 2-h of serum starvation, cells were washed and cultured for 2 h in KRB in the presence or absence of atorvastatin and/or insulin 10^{-9} M; at the end of the incubation glucagon levels were measured. In control cells, glucagon secretion was significantly inhibited by insulin. In contrast, in cells pre-exposed to atorvastatin, insulin showed markedly reduced effect (Fig. 7).

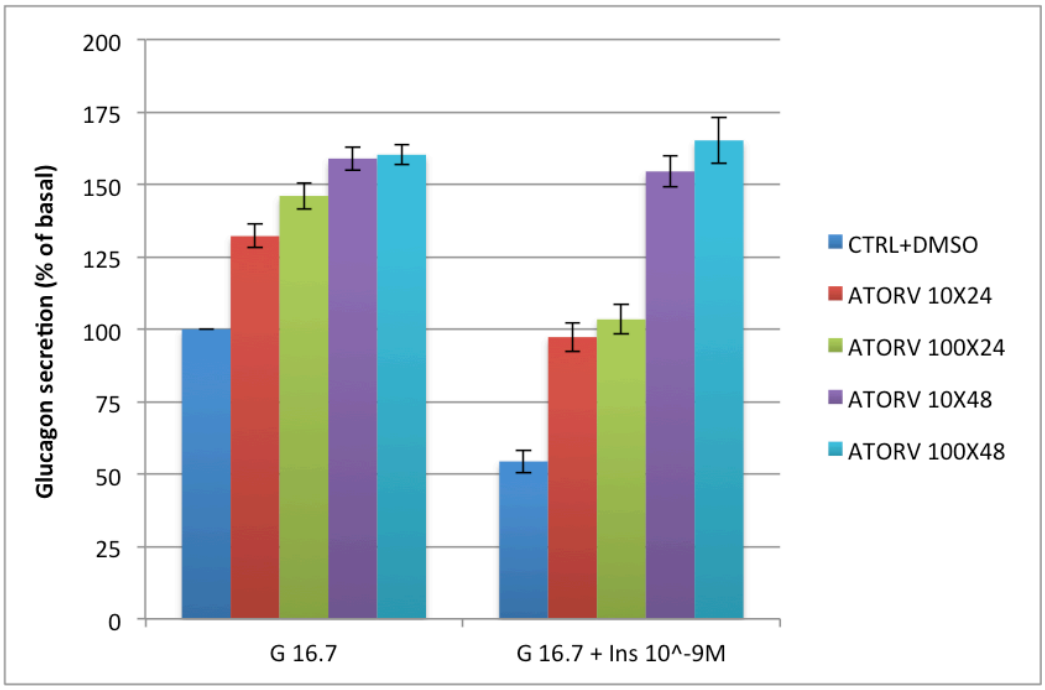

Figure 7: ***Effect of atorvastatin (10 or 100 ng/ml for 24 or 48h) on glucagon secretion in αTC1-C6 cells.***

CONCLUSIONS

In this study, we provide evidence that the chronic exposure to atorvastatin but not to pravastatin impairs glucose-induced secretion of insulin and GLP-1 in *in vitro* models of respectively pancreatic β-cells (INS-1) and intestinal L-cells (GLUTag). In addition, we observed that atorvastatin caused a decrease in ATP synthesis in INS1 cells. Interestingly, pravastatin, a hydrophilic statin, affected neither insulin secretion nor ATP production in INS-1 cells.

Moreover, to evaluate the ability of atorvastatin to induce a state of insulin-resistance at the level of pancreatic alpha cells, we investigated the insulin ability to suppress glucagon release. We found that cells exposed to atorvastatin release more glucagon at the basal culture condition. Additionally, in these cells, the inhibitory effect of insulin on glucagon secretion was blunted and IRS-1 phosphorylation was reduced.

These data provide new and useful information regarding the underlying mechanism of the statin-related diabetes risk. Specifically, our results appear to indicate that the diabetogenic effect of statins could be related to both impaired insulin release, due to reduced insulin secretion and ATP production or to the impairment of GLP-1 stimulation, and incresed insulin-resistance in an insulin target tissue as the pancreatic alpha cell.

BIBLIOGRAPHY

[1] Thom S, Rodgers A (2014) Combination pill for cardiovascular disease--reply. JAMA : the journal of the American Medical Association 311: 93-94
[2] Mendis S (2010) The contribution of the Framingham Heart Study to the prevention of cardiovascular disease: a global perspective. Progress in cardiovascular diseases 53: 10-14
[3] Haffner SM, Lehto S, Ronnemaa T, Pyorala K, Laakso M (1998) Mortality from coronary heart disease in subjects with type 2 diabetes and in nondiabetic subjects with and without prior myocardial infarction. The New England journal of medicine 339: 229-234
[4] Cholesterol Treatment Trialists C, Baigent C, Blackwell L, et al. (2010) Efficacy and safety of more intensive lowering of LDL cholesterol: a meta-analysis of data from 170,000 participants in 26 randomised trials. Lancet 376: 1670-1681
[5] Ridker PM, Danielson E, Fonseca FA, et al. (2009) Reduction in C-reactive protein and LDL cholesterol and cardiovascular event rates after initiation of rosuvastatin: a prospective study of the JUPITER trial. Lancet 373: 1175-1182
[6] Davignon J (2004) Beneficial cardiovascular pleiotropic effects of statins. Circulation 109: III39-43
[7] Bellosta S, Paoletti R, Corsini A (2004) Safety of statins: focus on clinical pharmacokinetics and drug interactions. Circulation 109: III50-57
[8] Ridker PM, Danielson E, Fonseca FA, et al. (2008) Rosuvastatin to prevent vascular events in men and women with elevated C-reactive protein. The New England journal of medicine 359: 2195-2207
[9] Ridker PM, Pradhan A, MacFadyen JG, Libby P, Glynn RJ (2012) Cardiovascular benefits and diabetes risks of statin therapy in primary prevention: an analysis from the JUPITER trial. Lancet 380: 565-571
[10] Freeman DJ, Norrie J, Sattar N, et al. (2001) Pravastatin and the development of diabetes mellitus: evidence for a protective treatment effect in the West of Scotland Coronary Prevention Study. Circulation 103: 357-362
[11] Koh KK, Quon MJ, Han SH, Lee Y, Kim SJ, Shin EK (2010) Atorvastatin causes insulin resistance and increases ambient glycemia in hypercholesterolemic patients. Journal of the American College of Cardiology 55: 1209-1216
[12] Waters DD, Ho JE, DeMicco DA, et al. (2011) Predictors of new-onset diabetes in patients treated with atorvastatin: results from 3 large randomized clinical trials. Journal of the American College of Cardiology 57: 1535-1545
[13] LaRosa JC, Grundy SM, Waters DD, et al. (2005) Intensive lipid lowering with atorvastatin in patients with stable coronary disease. The New England journal of medicine 352: 1425-1435
[14] Pedersen TR, Faergeman O, Kastelein JJ, et al. (2005) High-dose atorvastatin vs usual-dose simvastatin for secondary prevention after myocardial infarction: the IDEAL study: a randomized controlled trial. JAMA : the journal of the American Medical Association 294: 2437-2445
[15] Sever PS, Dahlof B, Poulter NR, et al. (2003) Prevention of coronary and stroke events with atorvastatin in hypertensive patients who have average or lower-than-average cholesterol concentrations, in the Anglo-Scandinavian Cardiac Outcomes Trial--Lipid Lowering Arm (ASCOT-LLA): a multicentre randomised controlled trial. Lancet 361: 1149-1158
[16] McCarey DW, McInnes IB, Madhok R, et al. (2004) Trial of Atorvastatin in Rheumatoid Arthritis (TARA): double-blind, randomised placebo-controlled trial. Lancet 363: 2015-2021
[17] Paul F, Waiczies S, Wuerfel J, et al. (2008) Oral high-dose atorvastatin treatment in relapsing-remitting multiple sclerosis. PloS one 3: e1928

[18] Martin S, Herder C, Schloot NC, et al. (2011) Residual beta cell function in newly diagnosed type 1 diabetes after treatment with atorvastatin: the Randomized DIATOR Trial. PloS one 6: e17554
[19] Kjekshus J, Apetrei E, Barrios V, et al. (2007) Rosuvastatin in older patients with systolic heart failure. The New England journal of medicine 357: 2248-2261
[20] Keech A, Colquhoun D, Best J, et al. (2003) Secondary prevention of cardiovascular events with long-term pravastatin in patients with diabetes or impaired fasting glucose: results from the LIPID trial. Diabetes care 26: 2713-2721
[21] Study of the Effectiveness of Additional Reductions in C, Homocysteine Collaborative G, Armitage J, et al. (2010) Intensive lowering of LDL cholesterol with 80 mg versus 20 mg simvastatin daily in 12,064 survivors of myocardial infarction: a double-blind randomised trial. Lancet 376: 1658-1669
[22] Koh KK, Quon MJ, Han SH, et al. (2008) Simvastatin improves flow-mediated dilation but reduces adiponectin levels and insulin sensitivity in hypercholesterolemic patients. Diabetes care 31: 776-782
[23] Culver AL, Ockene IS, Balasubramanian R, et al. (2012) Statin use and risk of diabetes mellitus in postmenopausal women in the Women's Health Initiative. Archives of internal medicine 172: 144-152
[24] Goldstein MR, Mascitelli L (2013) Do statins cause diabetes? Current diabetes reports 13: 381-390
[25] Axsom K, Berger JS, Schwartzbard AZ (2013) Statins and diabetes: the good, the bad, and the unknown. Current atherosclerosis reports 15: 299
[26] Sattar N, Taskinen MR (2012) Statins are diabetogenic--myth or reality? Atherosclerosis Supplements 13: 1-10
[27] Brault M, Ray J, Gomez YH, Mantzoros CS, Daskalopoulou SS (2014) Statin treatment and new-onset diabetes: a review of proposed mechanisms. Metabolism: clinical and experimental 63: 735-745
[28] Piro S, Maniscalchi ET, Monello A, et al. (2010) Palmitate affects insulin receptor phosphorylation and intracellular insulin signal in a pancreatic alpha-cell line. Endocrinology 151: 4197-4206