Relazione Borsa di Studio FO.DI.RI.-MSD 2015/2016

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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. The diabetogenic effect of statins seems to be directly related to the dose of statins, to the degree of attained LDL cholesterol lowering [27] and to the hydrophilic or lipophilic nature of statins.

The benefits of statin therapy in reducing CVD events far outweigh the diabetes hazard [9, 28, 29]; nevertheless, it is important to deeply understand the molecular mechanisms through which these compounds affect glucose homeostasis.

These mechanisms could potentially involve an increased insulin resistance, a decreased β-cell function or a combination of these two processes [30]

OBJECTIVE

Since in the first year of the project (Borsa di Studio FO.Di.RI 2014/2015) we have demonstrated that the chronic exposure to atorvastatin but not to pravastatin impairs insulin secretion and ATP production, in the second year of the project we tried to deeply investigate the molecular mechanisms involved in the onset of these alterations. In particular, we investigated mitochondrial function and ROS production in models of pancreatic β-cells chronically treated with statins.

Moreover, since the inhibition of the HMG-CoA conversion to mevalonate suppressed not only the synthesis of cholesterol, but also of other intermediates, such as Coenzyme Q10 (CoQ10), we also investigated CoQ10 modulation and mevalonate co-treatment effect in our system.

MATERIALS AND METHODS

Cell models

For our study we used the rat INS-1 cell line (pancreatic β-cells model), a kind gift from C. B. Wollheim, University of Geneva, Geneva, Switzerland.

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 INS-1 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). 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.).

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.

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

OxPhos complexes expression in INS-1 cells after atorvastatin treatment

Because ATP synthesis depends on the transfer of electrons through the mitochondrial respiratory chain complexes I–IV and on the phosphorylation of ADP into ATP by the Complex V, we studied respiratory chain complexes expression, by Western blot, in INS-1 cells chronically (24 or 48h) treated with atorvastatin (10 or 100 ng/mL).

We found that the protein levels of the complexes were significantly decreased in cells that had been pre-exposed to atorvastatin compared to control cells with a maximal effect at the highest dose-time combination (100 ng/mL for 48 h) (Fig. 1).

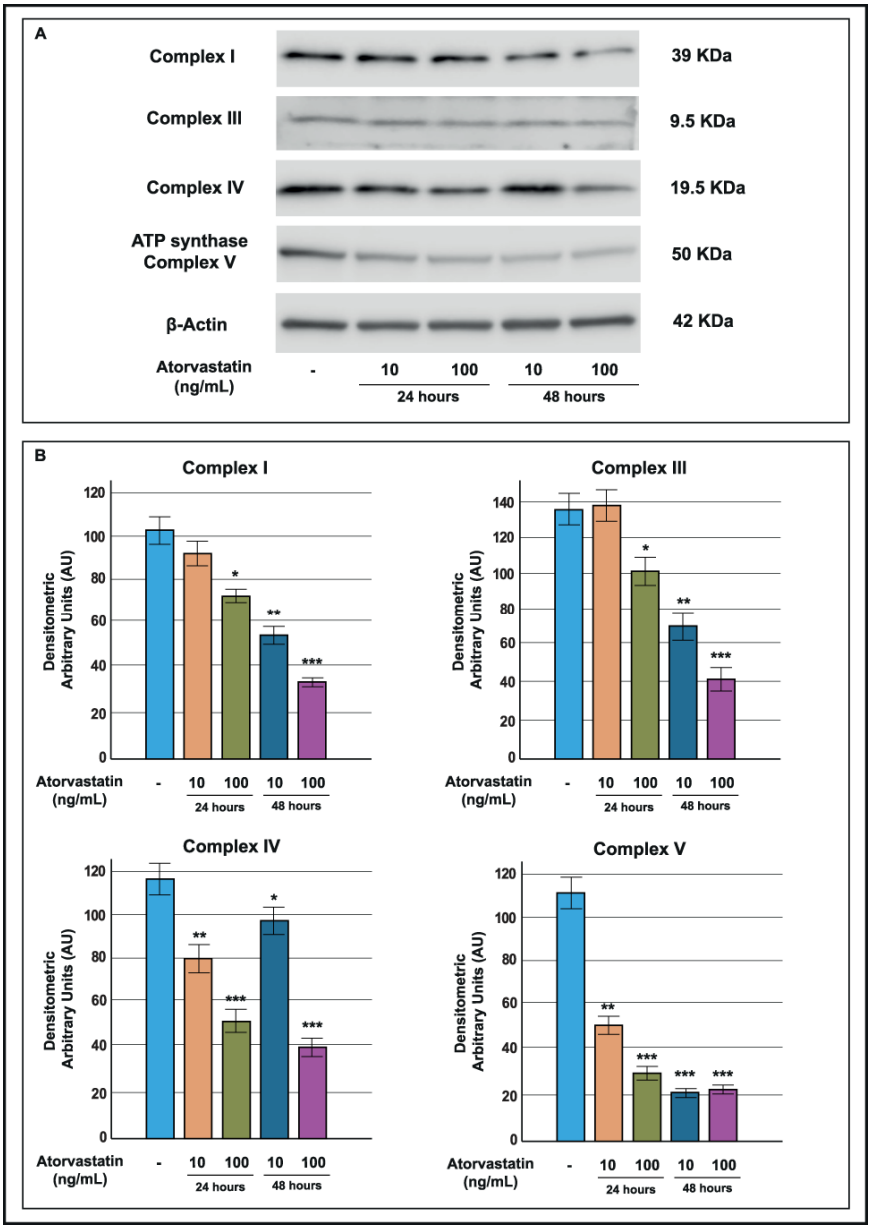

Figure 1: ***Effect of pre-exposure to atorvastatin on protein expression of mitochondrial respiratory chain complexes in INS-1 cells***

Atorvastatin increased ROS production in INS-1 cells

Since statins have been shown to increase intracellular reactive oxygen species in different models, we measured oxygen free radical production in cells cultured for 24 or 48h with atorvastatin (10 or 100 ng/mL) and found that the treatment produced a significant increase of ROS levels in a dose-time dependent manner (Fig. 2).

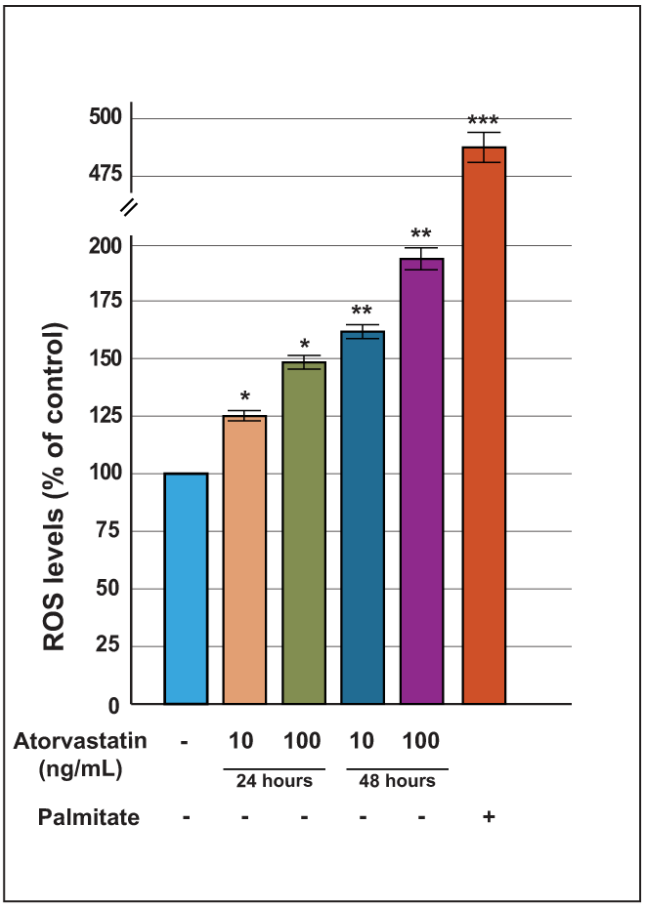

Figure 2: ***Effect of pre-exposure to atorvastatin on reactive oxygen species (ROS) production in INS-1 cells***

Insulin secretion and OxPhos complexes expression in INS-1 cells after atorvastatin and mevalonate co-treatment

Statins act by competitively inhibiting HMG-CoA reductase activity and blocking the conversion of HMG-CoA to mevalonate.

In order to determine whether the impairments induced by atorvastatin resulted from the inhibition of the mevalonate pathway, we added increasing concentrations of mevalonate (50, 100, 500 or 1000 µM) to INS-1 cells simultaneously treated with 100 ng/mL atorvastatin for 48 h. As evidenced by Western Blot analysis, mevalonate increased the expression of the mitochondrial complexes with a statistically significant effect for all the proteins at the concentration of 500 µM (Fig. 3).

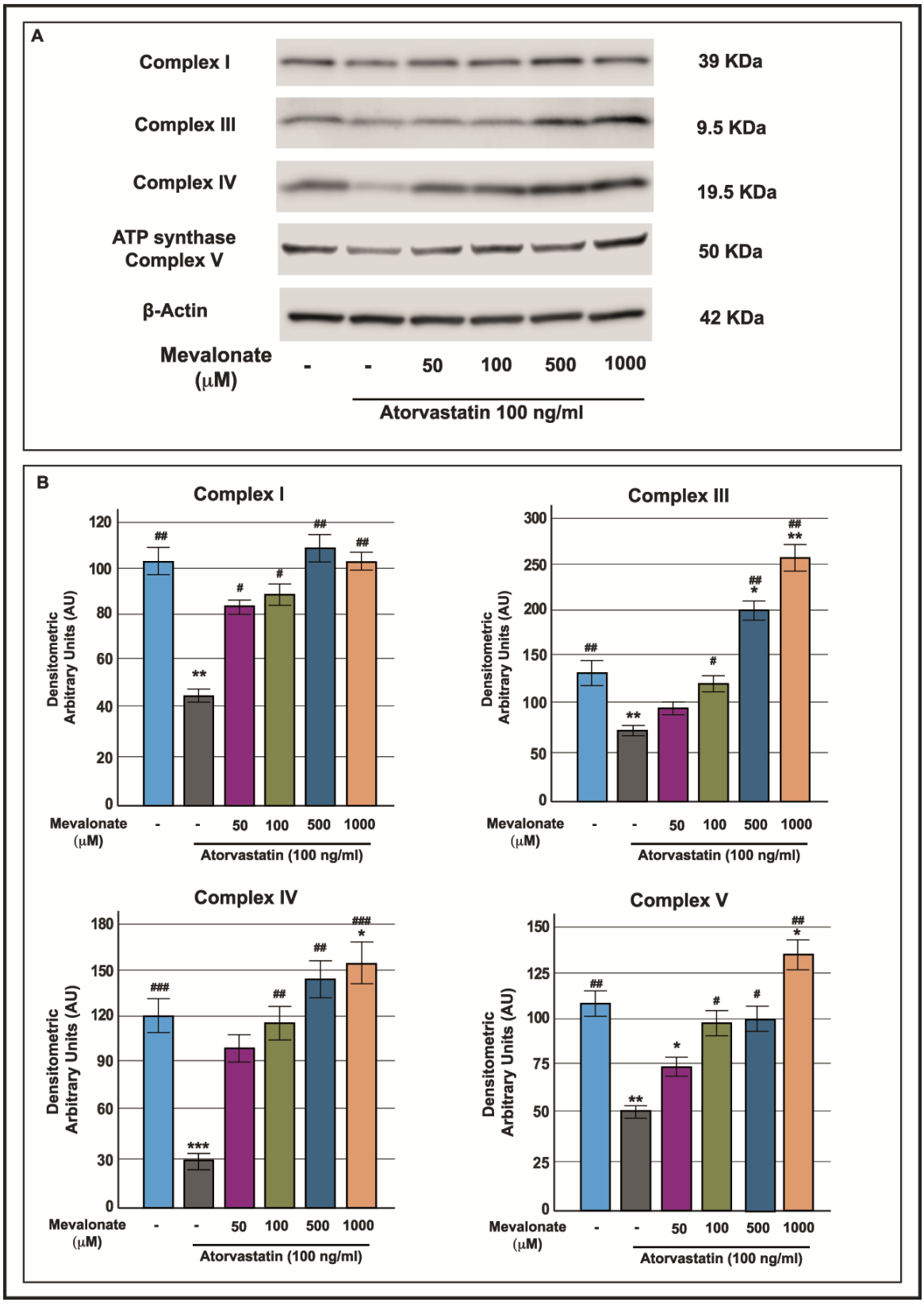

Figure 3: *Effect of mevalonate addition, in INS-1 cells simultaneously exposed to atorvastatin, on protein expression of mitochondrial respiratory chain complexes*

On the basis of these findings, we evaluated the effect of the addition of 500 μM mevalonate to INS-1 cells treated at the same time with atorvastatin (100 ng/mL for 48 h) on acute glucose stimulated

insulin secretion and observed that the secretory defects induced by atorvastatin in INS-1 cells were completely prevented by the contemporary presence of mevalonate (Fig. 4).

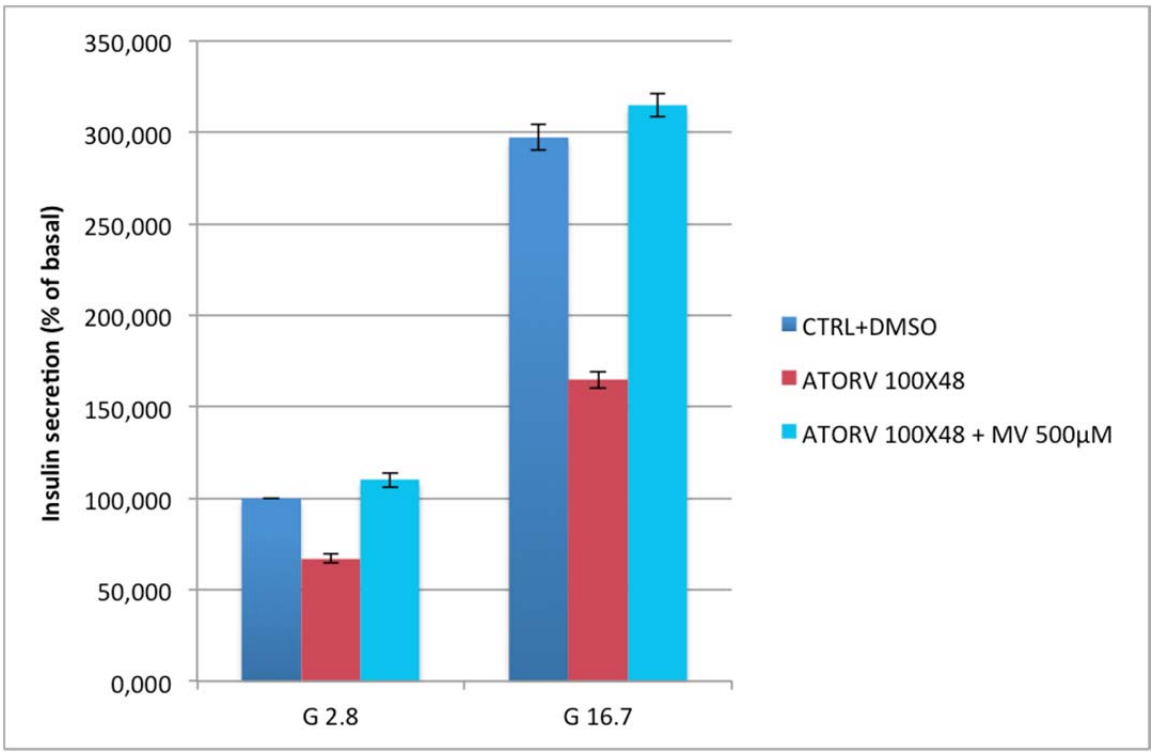

Figure 4: ***Effect of mevalonate addition in INS-1 cells simultaneously exposed to atorvastatin, on glucose-induced insulin release***

Coenzyme Q10 expression in INS-1 cells after atorvastatin treatment and mevalonate addition

Because mevalonate also constitutes a precursor of Coenzyme Q10 or ubiquinone, we also measured the levels of this protein in INS-1 cells chronically (24 or 48h) treated with atorvastatin (10 or 100 ng/mL). We observed a significant decrease in ubiquinone expression in atorvastatin-treated cells compared with control cells, as evaluated by Western Blot analysis.

On these bases, we studied the expression of coenzyme Q after the addition of increasing concentrations of mevalonate (50, 100, 500 or 1000 µM) to INS-1 cells contemporarily treated with 100 ng/mL atorvastatin for 48 h. As evidenced by Western Blot analysis, mevalonate increased the expression of ubiquinone with a statistically significant effect at the concentration of 500 µM (Fig. 5).

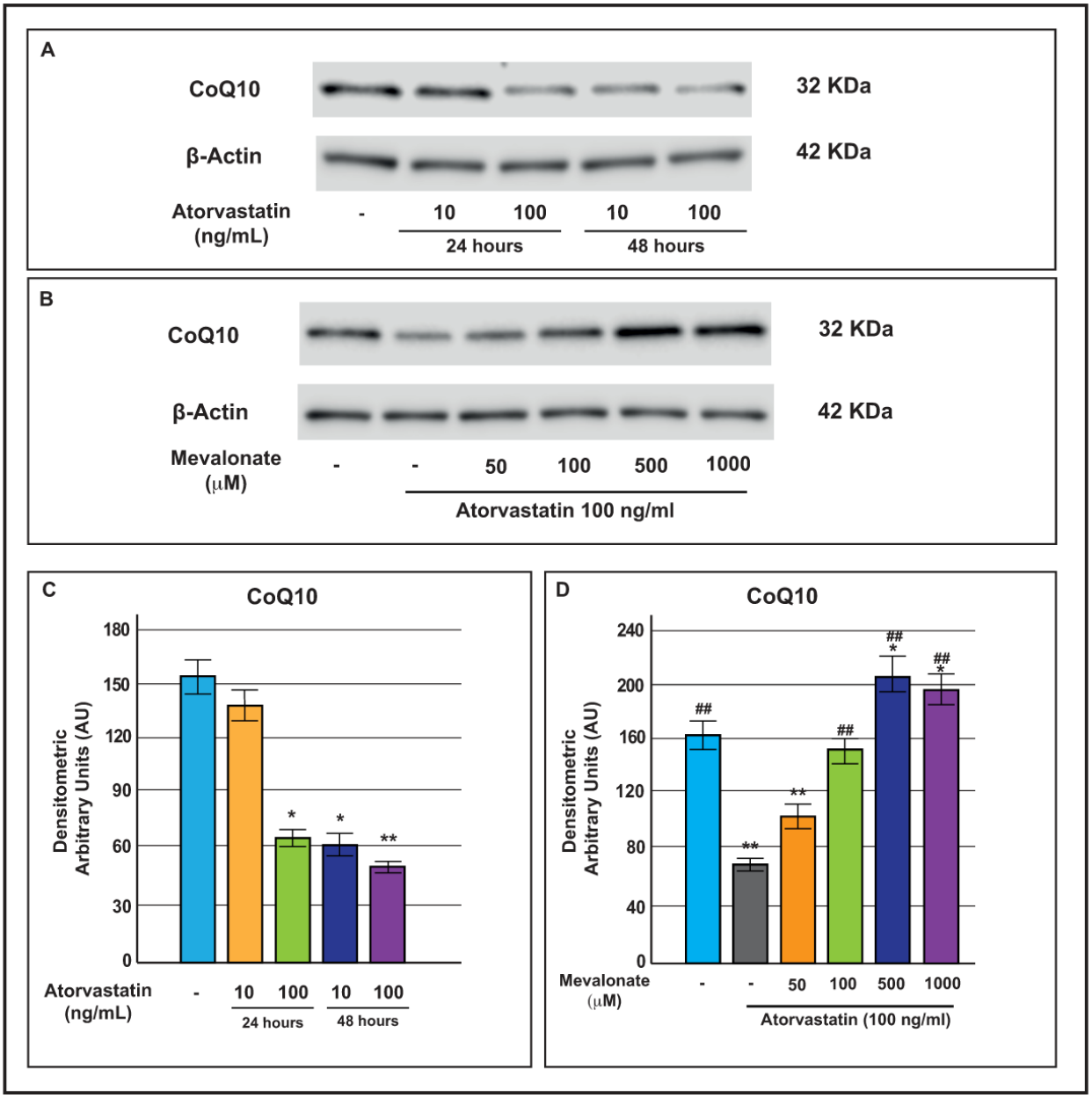

Figure 5: *Effect of exposure to atorvastatin and co-treatment with mevalonate on protein expression of Coenzyme Q10 (CoQ10) in INS-1 cells*

CONCLUSIONS

In this work, we provide evidence that chronic exposure to atorvastatin (lipophilic statin), but not to pravastatin (hydrophilic statin), inhibited both basal and glucose-stimulated insulin secretion in INS-1 β-cells in a dose-time dependent manner. In addition, we demonstrate that this secretory alteration is associated with mitochondrial dysfunctions induced by conditions of oxidative stress.

These data provided a new molecular mechanism behind the dysregulation of β-cell secondary to statin exposure. The key point of our results is, instead, the mitochondria; in pancreatic β-cells, with low antioxidant capacities, atorvastatin decreased CoQ10 levels and induced high oxidative stress, responsible for mitochondrial deterioration.

In our study, mevalonate co-treatment allowed replenishment of CoQ10 and oxphos complexes, preventing statin-induced β-cell dysfunction. These results may suggest that the decrease in CoQ10 level and the consequent high oxidative stress, in a limited ROS-scavenging system, could be the triggering factor inducing mitochondrial dysfunction and down-regulation of insulin secretion.

In conclusion, the present study supports evidences regarding the different diabetogenicity of lipophilic and hydrophilic statins and demonstrates a direct deleterious effect of these drugs on mitochondria due to the suppression of the antioxidant defense system and induction of ROS production in a model of human pancreatic β-cells.

This may have important clinical implications because it indicates the existence of a novel mechanism linking treatment with lipophilic statins to the increased risk of type 2 diabetes. It also suggests that coenzyme Q may be a potential target for preserving β-cell function during statin-therapy. Finally, these data may help to design strategies for prevention or reduction of statin induced β-cell dysfunction and diabetes in patients treated with lipophilic statins.

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