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Original research article

Exenatide prevents statin-related LDL receptor increase and improves insulin secretion in pancreatic beta cells (1.1E7) in a protein kinase A-dependent manner

Łukasz Bułdak [1] *, Grzegorz Machnik [1], Estera Skudrzyk [1], Aleksandra Bołdys [1], Mateusz Maligłówka [1], Michał Kosowski [1], Marcin Basiak [1], Rafał Jakub Bułdak [2], Bogusław Okopień [1]

[1] *Medical University of Silesia, School of Medicine in Katowice, Department of Internal Medicine and Clinical Pharmacology, Katowice, Poland*
[2] *University of Opole, Institute of Medical Sciences, Opole, Poland*

Abstract
Statins are primary drugs in the treatment of hyperlipidemias. This group of drugs is known for its beneficial pleiotropic effects (e.g., reduction of inflammatory state). However, a growing body of evidence suggests its diabetogenic properties. The culpable mechanism is not completely understood and might be related to the damage to pancreatic beta cells. Therefore, we conceived an *in vitro* study to explore the impact of atorvastatin on pancreatic islet beta cells line (1.1.E7). We evaluated the influence on viability, insulin, low-density lipoprotein (LDL) receptor, and proprotein convertase subtilisin/kexin type 9 (PCSK9) expression. A significant drop in mRNA for proinsulin and insulin expression was noted. Concurrently, a rise in LDL receptor at the protein level in cells exposed to atorvastatin was noted. Further experiments have shown that exenatide – belonging to glucagon-like peptide 1 (GLP-1) analogs that are used in a treatment of diabetes and known for its weight reducing properties – can alleviate the observed alterations. In this case, the mechanism of action of exenatide was dependent on a protein kinase A pathway. In conclusion, our results support the hypothesis that statin may have diabetogenic properties, which according to our study is related to reduced insulin expression. The concomitant use of GLP-1 receptor agonist seemed to successfully revert insulin expression.

Keywords: Atorvastatin; Beta islet cells; Diabetes; GLP-1; *In vitro*; Insulin; LDL receptor; PCSK9; Pleiotropic effects

Highlights:
- Atorvastatin shows diabetogenic effects by reducing insulin expression.
- Atorvastatin increases the LDL-R in pancreatic beta cells, which might lead to lipotoxicity.
- Exenatide reverses the impact of atorvastatin on beta cells in a PKA dependent manner.

Abbreviations:
1.1E7 – human pancreatic beta cell line; BCA – bicinchoninic acid assay; BSA – bovine serum albumin; DM2 – type 2 diabetes mellitus; GAPDH – glyceraldehyde 3-phosphate dehydrogenase; GLP-1 – glucagon-like peptide-1; HMG-CoA – hydroxymethylglutaryl-CoA; INS-1 – rat insulinoma cell line; LDL – low density lipoprotein; n.s. – not significant; p-CREB – phosphorylated cAMP response element binding; PCSK9 – proprotein convertase subtilisin/kexin type 9; PKA – protein kinase A; PVDF – polyvinylidene fluoride

Introduction

Hypercholesterolemia is a major risk factor in the development of atherosclerosis and premature cardiovascular deaths (Verma and Brinton, 2014). Statins lead to improvements in patients with hypercholesterolemia (Yebyo et al., 2019). The primary mechanism of statins' action relies on the inhibition of hydroxymethylglutaryl-CoA (HMG-CoA) reductase. Their use indirectly lead to an increase in the expression of low-density lipoprotein (LDL) receptors, resulting in the accumulation of cholesterol in certain organs (e.g., pancreatic beta cells, muscles, adipose tissue), which might cause oxidative stress and cellular damage. Furthermore, statins lead to a reflex elevation in proprotein convertase subtilisin/kexin type 9 (PCSK9) expression (Yu et al., 2017). PCSK9 is a protein preventing LDL-R from resurfacing on cell membranes, resulting in reduced clinical effectiveness of statins and leading to certain adverse effects. Mutations in PCSK9 were shown to be responsible for familial hypercholesterolemia. However, in contrast to familial hypercholesterolemia resulting from *LDL-R* loss-of-function mutations, they are not connected with re-

* **Corresponding author:** Łukasz Bułdak, Medical University of Silesia, School of Medicine in Katowice, Department of Internal Medicine and Clinical Pharmacology, Medykow 18, 40-752 Katowice, Poland; e-mail: lbuldak@gmail.com
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duced propensity toward type 2 diabetes mellitus (DM2) (Tada et al., 2016). Long term use of HMG-CoA inhibitors accelerate diabetes development (Khan et al., 2019). The existence of a clinically important relation between PCSK9 level and the risk of development of DM2 is suspected. Nevertheless, patients with DM2, still benefit from lipid lowering therapy, to an even greater extent than patients without DM2 (Zhang et al., 2012). Therefore, International Societies recommend more stringent control of lipid profile in diabetic subjects (Visseren et al., 2021).

Several mechanisms might be responsible for impaired glucose metabolism during statin therapy. HMG-CoA reductase inhibition reduced beta cell mass in an *in vivo* mice model (Takei et al., 2020) and diminished insulin synthesis in INS-1 cells (Sun et al., 2016). Lovastatin reduced insulin sensitivity in cultured adipocytes, which was reflected by reduced expression of insulin dependent GLUT-4 glucose transporters (Chamberlain, 2001).

Pancreatic islets beta cell depletion and loss of its function is seen in most patients with DM2 (Halban et al., 2014). Novel antidiabetic drugs, GLP-1 analogs, seem to prevent beta cell depletion and improve beta cell function (Anholm et al., 2019). Additionally, GLP-1 analogs were recently approved for the treatment of obesity (FDA, 2022), which is essential in patients with hypercholesterolemia and DM2. Furthermore, weight loss might be responsible for lipid lowering properties during GLP-1 treatment (Aoki et al., 2020). Beta islet cells are fragile and prone to oxidative stress. Exenatide, a GLP-1 analog, was able to protect beta islet cells 1.1E7 against oxidative stress introduced by hydrogen peroxide (Bułdak et al., 2022). Increased cholesterol uptake during lipid lowering therapies may also lead to aggravated lipotoxicity (Panajatovic et al., 2021). Furthermore, PCSK9 itself may be involved in many more pathological pathways than simply the impact on cholesterol level (Maligłówka et al., 2021). The influence of statin on PCSK9 expression and its potential involvement in the development of pancreatic beta cells has not been explored.

There is little data that explores the potential cause of deteriorating glucose control during statin therapy which focuses on experiments on human pancreatic beta cells in *in vitro* conditions. GLP-1 analogs are established therapies in DM2 and seem to have protective actions in pancreatic islets beta cells. Furthermore, there are no data on the potential effects actions of GLP-1 analogues on the function of those cells treated with statin. Therefore, we conceived a study to explore the impact of atorvastatin on cell viability and insulin expression in 1.1E7 human pancreatic beta cell line. Additionally, we explored whether such an impact may be related to alterations in the expression of LDL-R and PCSK-9. Finally, we evaluated the influence of exenatide, a GLP-1 analogue, on the 1.1E7 cells that were exposed to atorvastatin.

Materials and methods

Cell culture

Human pancreatic beta cell line (1.1E7) was acquired from the European Collection of Authenticated Cell Cultures (ECACC), distributed by Merck Sigma-Aldrich (Poznań, Poland). Cells were cultured in the RPMI-1640 with 2 mM Glutamine supplemented with 10% FBS and glucose at a final concentration of 100 mg/dl or 400 mg/dl. On the day of the experiment, culture medium was supplemented with reagents depending on the experimental setting to reach the final concentration: exenatide (Exendin-4, Cat. No. E7144, Merck Sigma-Aldrich, Poznań, Poland) – 10 nM solution; atorvastatin (Cat. No. PZ0001, Merck Sigma-Aldrich, Poznań, Poland) – 10 ng/ml or 100 ng/ml and PKI (14-22) (Cat. No. 476485, Merck Sigma-Aldrich, Poznań, Poland) – 10 μM. 1.1E7 cells were incubated at 37 °C in an atmosphere containing 95% air and 5% CO_2 in a CO_2 incubator (Heracell, Thermo Fisher Scientific, Inc., Grand Island, NY, USA). Experiments were performed under standard conditions for 24 hours. Afterwards samples were collected and stored as described below.

Viability

Trypan blue method (0.4%) was employed for the assessment of viability. TC-20 automated cell counter (Bio-Rad, Hercules, CA, USA) was used according to the manufacturer's instruction. Cell suspension (10 μl) was mixed with aliquots of 0.4% trypan blue (10 μl) and incubated for 3 min. Afterwards, the sample was put on a slide and processed in the automated counter. Finally, cell concentration in a sample and a percentage of viable cells were obtained. Experiments were performed in duplicates.

RT-qPCR

The assessment of proinsulin, LDL receptor and PCSK9 was performed using quantitative polymerase chain reaction (qPCR). Primer sequence for proinsulin was obtained from PrimerBank (https://pga.mgh.harvard.edu/primerbank/ – GenBank Accession NM_000207): forward – GCAGCCTTTGTGAACCAACAC; reverse – CCCCGCACACTAGGTAGAGA. Whereas sequences for LDL-R and PCSK9 were identified in previous studies (Dong, 2010). LDLR: forward – GACGTGGCGTGAACATCTG; reverse – CTGGCAGGCAATGCTTTGG. PCSK9: forward – AGGGGAGGACATCATTGGTG; reverse – CAGGTTGGGGGTCAGTACC. Glyceraldehyde 3-phosphate dehydrogenase gene (GAPDH) was selected as a reference gene for further calculations.

Total RNA was extracted from cultures of 1.1E7 cells by adding 1 ml of TRI reagent (MRC Inc. Cincinnati, OH, USA) to lyse cells derived from one of 12-well culture plates (1×10^5 cells per well) (SPL Life Sciences Co., Ltd., Immuniq, Żory, Poland) according to manufacturer's recommendations. The final amount of RNA was dissolved in 150 μl of nuclease-free water. To remove potential contamination with DNA, samples containing 2 μg of total RNA were exposed to DNase-I (RNase-free, Life Technologies, Warsaw, Poland). Afterwards, 1 μg DNA-free RNA was reverse transcribed and suspended in 20 μl solution. In the final step of the procedure it was further diluted by factor 5 – as recommended by the manufacturer (GoScript Reverse Transcription System, Promega GmbH, Germany).

RT-PCR reaction mixtures consisted of 10 μl of SYBR Select Master Mix (Thermo Fisher Scientific), 0.2 μM of each primer (proinsulin F/R or LDL-R F/R or PCSK9 F/R), 2 μl of reverse transcription mixture (equivalent of 10 ng of total RNA). Roche LightCycler 480 Instrument II (Roche Diagnostics, Warsaw, Poland) with a specific thermal profile (94 °C/3 min then 35 cycles of 94 °C/30 s, 58 °C/30 s, 72 °C/45 s) was used to estimate the level of gene expression. Specificity of products was confirmed using the melting curve method. Increasing fluorescence was measured in real-time to obtain the value cycle threshold (CT), which was normalized to GAPDH expression and used for calculation of gene expression, according to $2^{(-\Delta\Delta Ct)}$ formula (Livak and Schmittgen, 2001).

Immunofluorescent imaging

Cultures destined for immunofluorescent imaging were performed on black 24-well plates (Zell-Kontakt GmbH, Noerten-Hardenberg, Germany). Cells were seeded with the density of 5×10^4 per well and were cultured until the cell confluence of about 80–90% was observed. Afterwards, cells were exposed for 24 h to culture media containing reagents as described in experimental conditions. To obtain an immunofluorescent image, a selection of primary antibodies were used: anti-insulin monoclonal antibody (Cat. No. 14-9769-82, Thermo Fisher Scientific, Warsaw, Poland, anti LDL receptor (LDLR) antibody (Cat. No. PA5-22976, Thermo Fisher Scientific, Warsaw, Poland). On completion of the experiment, cells were rinsed 1× with 1 ml of PBS for 5 min at RT and immediately fixed with cold methanol for at least 20 min at –20 °C or until subsequent analyses were performed. On the day of immunofluorescent imaging, cells were thawed and rinsed twice at RT with PBS for 5 min with shaking on the rocking plate. Cell permeabilization was achieved by double incubation in a solution containing 0.1% Triton X-100 resolved in PBS (Merck, Poznań, Poland) for 5 min under continuous mixing, followed by washing the cells with PBS (5 min at RT). Unspecific binding sites were blocked by incubation of the cells for 40 min in a blocking solution containing 3% bovine serum albumin (BSA) diluted in PBS (Albumin Fraction V, Carl Roth GmbH, Germany, distributed by Linegal Chemicals, Warsaw, Poland). Meanwhile, aliquots (500 µl) of appropriate primary antibody solutions (1 : 200) were prepared in the incubation medium (2% BSA, 0.1% Triton X-100 in PBS). In the next step, cells were incubated with antibodies for 16 h (overnight) at 4 °C. On the following day, primary antibody solutions were removed and cells were washed twice with 0.1% of Triton X-100 in PBS. Afterwards, aliquots (500 µl) of fluorescently-labelled, secondary antibodies solution (1 : 300) were added into the cells: Anti-Rabbit IgG (whole molecule)-FITC antibody produced in goat (Cat. No. F0382, Merck Sigma-Aldrich, Poznań, Poland) or Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 546 (Cat. No. A11003, Thermo Fisher Scientific, Warsaw, Poland). Then, secondary antibodies were added for 60 min at RT under protection from daylight, then washed twice with PBS/0.1% Triton X-100 and rinsed once with PBS. Finally, cells were washed again with PBS for 1 min and stained with 4',6-diamidino-2-phenylindole (DAPI) stain (Cat. No. D1306, Thermo Fisher Scientifics, Warsaw, Poland). The fluorescent images were examined and observed under Delta Optical IB-100 microscope equipped with epifluorescence module (Delta Optical, Nowe Osiny, Poland). Images were digitalized and quantified using ImageJ software (Abramoff et al., 2004).

Western blot

In the western blot analysis, human-specific antibodies were used for PCSK9 proprotein convertase: PCSK9 antibody (Cat. No. MA5-32843, Thermo Fisher Scientific, Warsaw, Poland). For a semi-quantitative analysis, a level of reference protein – glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was utilized (anti-GAPDH antibody produced in rabbit, No. Cat. G9545, Merck Sigma-Aldrich, Poznań, Poland).

Cell cultures were performed on 12-well culture plates (1×10^5 cells per well) (SPL Life Sciences Co., Ltd., Immuniq, Żory, Poland). On the conclusion of experiments, plates were placed on ice and cells were washed briefly with ice-cold PBS (500 µl). Protein extraction was completed using cold RIPA buffer (150 µl) enriched with 1.5 µl of Halt Protease Inhibitor Cocktail (1 : 100 v/v) per well (both chemicals from Thermo Scientific, Warsaw, Poland). Total protein content was measured in each sample by bicinchoninic acid assay (BCA Protein Assay Kit, Cat. No. 71285-M, Merck Sigma-Aldrich, Poznań, Poland) and total protein concentration was calculated according to the standard curve based on bovine serum albumin (BSA) solutions of known protein concentration (Thermo Scientific, Warsaw, Poland). Proteins from cell lysates were separated by electrophoresis in 10% polyacrylamide gel in the presence of Color Plus Prestained Protein Marker (Cat. No. P7711, New England Biolabs, Lab-Jot, Warsaw, Poland). Protein samples (20 µg) were loaded into gel slots. In the next step, proteins were electroblotted overnight onto PVDF membrane (Merck Millipore, Poznań, Poland) at 100 mA. Unspecific binding sites were blocked by incubation of the membranes in 3% bovine serum albumin (BSA) solution in Tris-buffered saline (1× TBS) for 2 h. Then membranes were placed in 3% BSA/1× TTBS (TBS supplemented with 0.05% of Tween-20) solution containing primary antibody against PCSK9 (1 : 1000). Afterwards, incubation was completed on a rocking platform for 2 h at RT. Next, two washes in TTBS for 10 min each were done. Finally, an Anti-rabbit IgG (whole molecule)-peroxidase antibody (Cat. No. A0545, Merck Sigma Aldrich, Poznań, Poland) was added (antibody dilution: 1 : 5,000 in 3% BSA/TTBS). Incubation was performed for one hour under continuous rocking. In the final step, membranes were washed thrice (2× TTBS for 5 min each and 1× TBS for 5 min). Chemiluminescent signal was developed using Pierce ECL Western Blotting Substrate (Cat. No. 32209, Thermo Scientific, Warsaw, Poland). Membranes were digitalized using ChemiDoc-It Imaging System (Analytik Jena, Jena, Germany). Integrated optical density representing the level of the protein expression in a sample were performed using ImageJ software. Fig. 4 represents a set of 4 experiments.

Statistical analysis

The normality of distribution of data was evaluated using Shapiro–Wilk's test. All data were normally distributed and analyzed using one-way ANOVA with post-hoc Tukey test, and reported as means ± SEM. The p level below 0.05 was considered as statistically significant.

Results

Viability

Prior to further experiments, the impact of all conditions employed in our study have been assessed for the impact on viability of 1.1E7 cells. The results did not show any statistically significant changes in the viability of cultured cells (Fig. 1).

RT-PCR

Proinsulin mRNA expression

In hyperglycemic culture condition, transcriptional activity of proinsulin gene was not affected by exenatide (Fig. 2a). Similarly, at low concentration (10 ng/ml), atorvastatin was unable to show a statistically significant impact on proinsulin mRNA expression. However, a high concentration (100 ng/ml) of atorvastatin resulted in the diminished expression of proinsulin mRNA by 59% ($p < 0.05$).

Exenatide increased proinsulin expression that was previously reduced by the high concentration of atorvastatin, resulting in mRNA expression comparable similar to control samples. The effect was PKA dependent, which was shown in the experiment with PKI 14-22, leading to expression levels observed in cells exposed only to high concentration of atorvastatin in hyperglycemic conditions.

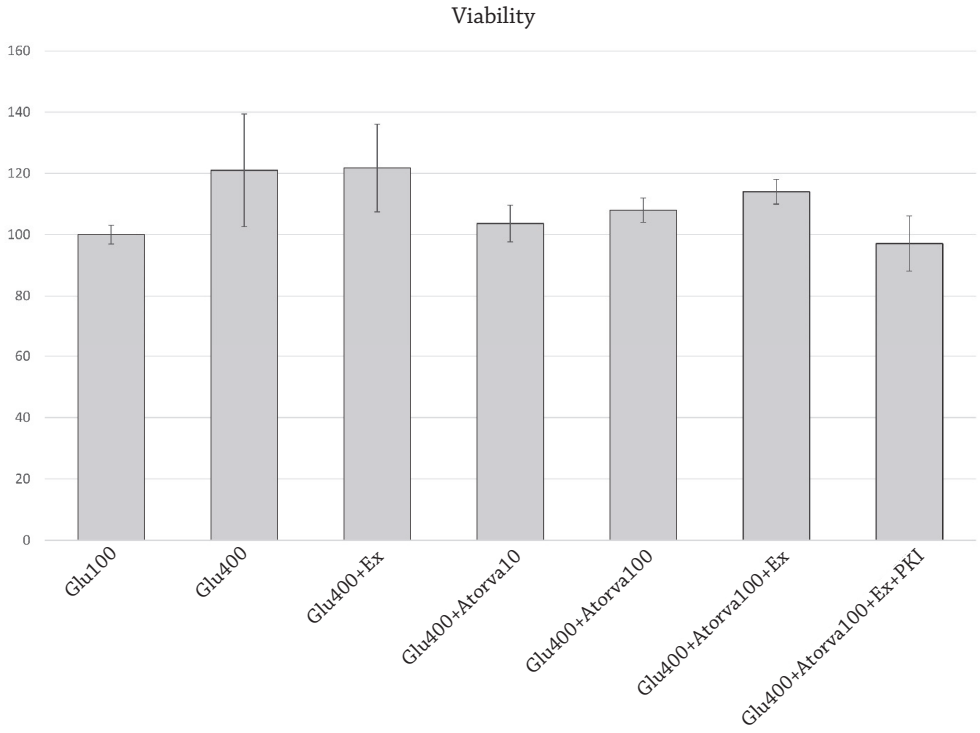
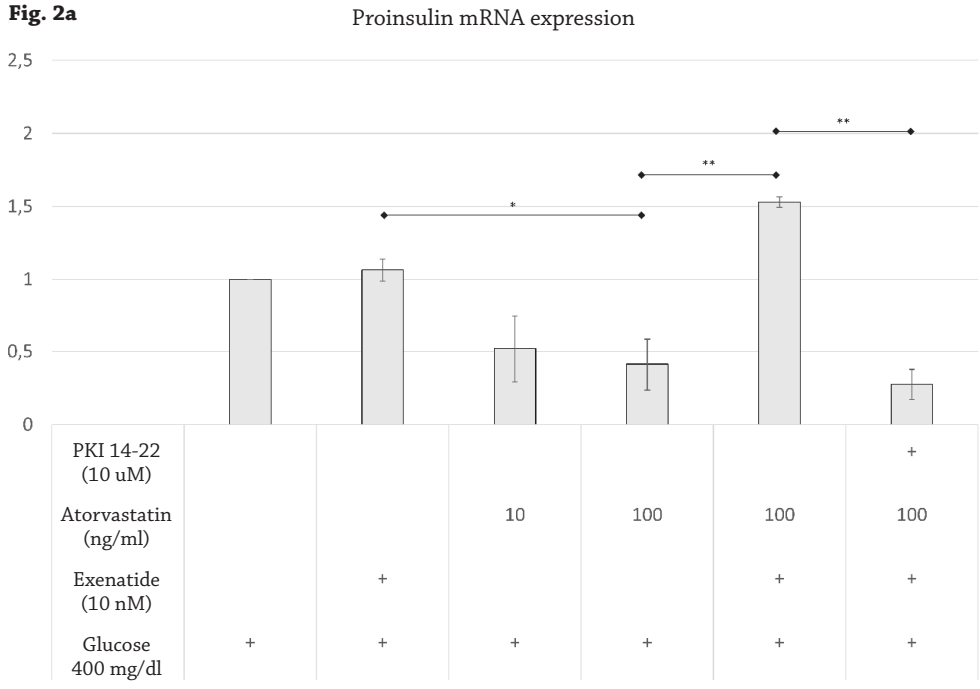


Fig. 1. Viability of 1.1E7 in experimental conditions

LDL mRNA expression
mRNA expression for LDL receptor was not affected by any experimental setting (Fig. 2b).

PCSK9 mRNA expression
The mRNA expression for PCSK9 was significantly reduced by 70% ($p < 0.05$) in cells exposed to high atorvastatin concentration (Fig. 2c). Exenatide prevented the reduction in mRNA expression that was noted in cells exposed high concentration of atorvastatin. Exenatide acted in a PKA dependent manner, which was shown in complete reversal of its effects by PKI 14-22.

Fig. 2a



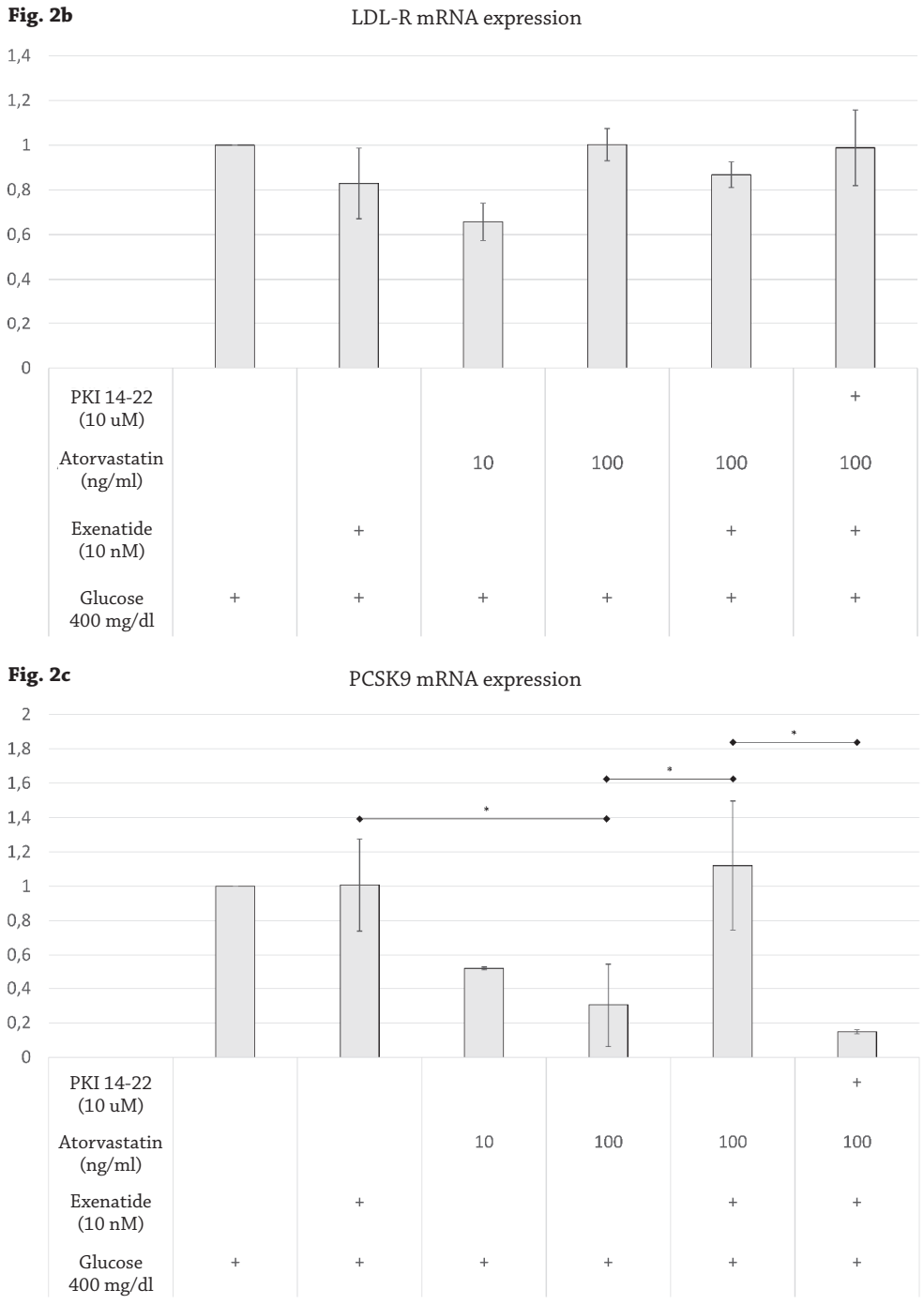


Fig. 2. The impact of atorvastatin and exenatide on the mRNA expression of proinsulin (**a**) and LDL-R (**b**) and PCSK9 (**c**). * <0.05; ** <0.01.

Immunostaining

Insulin expression

Compared to normoglycemia (100 mg/dl), hyperglycemic (400 mg/dl) culture conditions did not increase insulin expression in the cultures (Fig. 3b). However, in cells exposed to atorvastatin in hyperglycemic (400 mg/dl) conditions, the expression of insulin was lower than in cells exposed only to hyperglycemic environment. The lower atorvastatin concentration (10 ng/ml) resulted in a 23.1% ($p < 0.01$) reduction in insulin expression, whereas the higher concentration of atorvastatin (100 ng/ml) was associated with a 45% ($p < 0.01$) decrease in insulin level in 1.1E7 pancreatic beta cells. In the experimental setting, exenatide itself did not lead to an increase in the expression of insulin in hyperglycemic conditions (18%; p = n.s.). However, compared to cells treated in normoglycemic conditions, stimulation of cultured cells with both hyperglycemia and exenatide resulted in a significant increase in the expression of insulin (56%; $p < 0.05$).

In the next step of the experiments, we evaluated the impact of exenatide on insulin expression in cultures subjected to atorvastatin (Fig. 3c). The GLP-1 receptor agonist abolished the negative impact of high concentration atorvastatin

on pancreatic beta cells, resulting in insulin levels even higher than cultures exposed only to hyperglycemic environment (633 vs. 467 kAU; $p < 0.01$), and similar to those noted in cells exposed to hyperglycemia and exenatide (633 vs. 572 kAU; p = n.s.). The effect of exenatide was dependent on protein kinase A (PKA) signaling, which was shown in the experiment with PKI 14-22 (PKA inhibitor). PKI 14-22 resulted in a 31% ($p < 0.01$) reduction in insulin expression in cultures containing high concentration of atorvastatin and exenatide in hyperglycemic medium.

LDL receptor expression

Compared to cells cultured in a medium containing 100 mg/dl of glucose, LDL-R expression did not change in hyperglycemic cultures nor in hyperglycemic cultures with exenatide (Fig. 3d). Interestingly, the low concentration of atorvastatin was not connected with an increased expression of LDL receptors (754 vs. 1,065 kAU; p = n.s). However, in cultures exposed to a high concentration of atorvastatin, LDL receptors expression was significantly elevated in 1.1E7 pancreatic beta cells by 136% ($p < 0.01$).

Despite the lack of statistically significant influence of exenatide on LDL-R expression in pancreatic beta cells in normoglycemic and hyperglycemic culture conditions, we performed an experiment exploring the potential influence of GLP-1 analogs on LDL-R expression in cells exposed to atorvastatin (Fig. 3e). The acquired data showed that exenatide prevented the increase in LDL-R expression caused by atorvastatin at high concentration (1,780 vs. 813 kAU; $p < 0.01$), leading to levels comparable to those seen in cells subjected only to hyperglycemic conditions (813 vs. 754 kAU; p = n.s.). Afterwards, it was shown that this phenomenon relied on a major intracellular pathway of GLP-1 receptor agonists (i.e. PKA), which was shown in the experiment employing PKI 14-22 (i.e. PKA signaling inhibition). The addition of PKA to a culture medium that contained exenatide (10 nM) and atorvastatin (100 ng/ml) led to a significant increase (by 63.5%; $p < 0.05$) in the expression of LDL receptor, leading to levels comparable to those seen in cultures with only high concentration of atorvastatin (1,329 vs. 1,781 kAU; p = n.s.).

Western blot analysis

PCSK9 expression

In the experiments estimating the protein expression of PCSK9, we did not note any significant impact in all experimental conditions (Fig. 4).

Fig. 3a

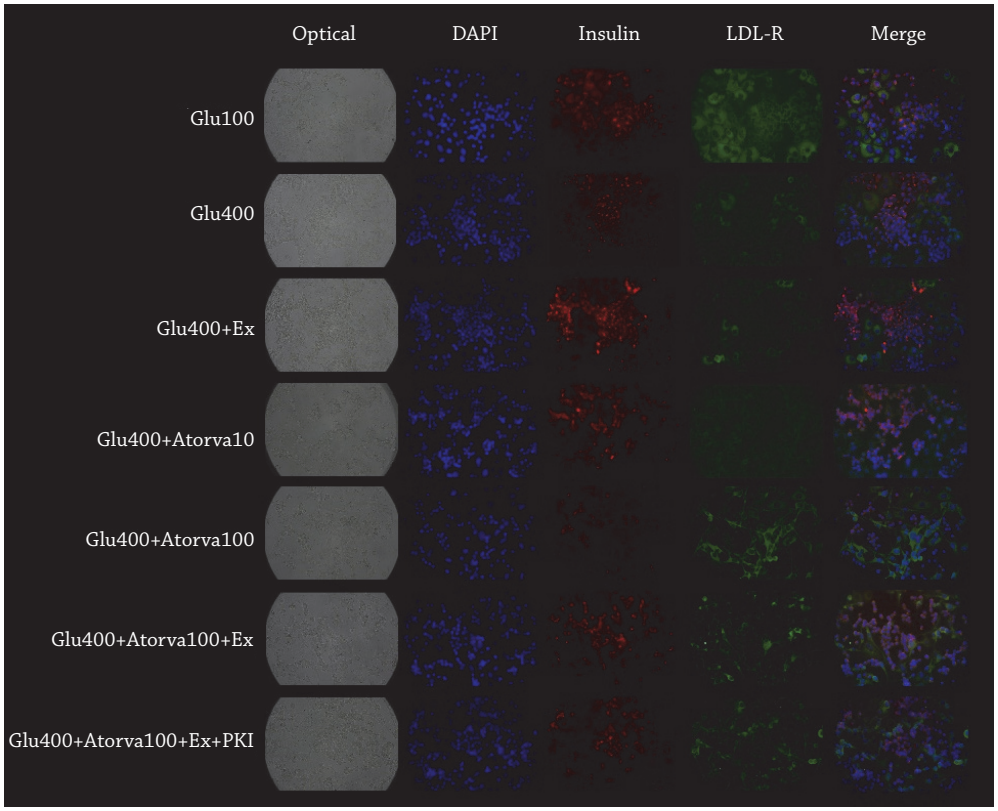
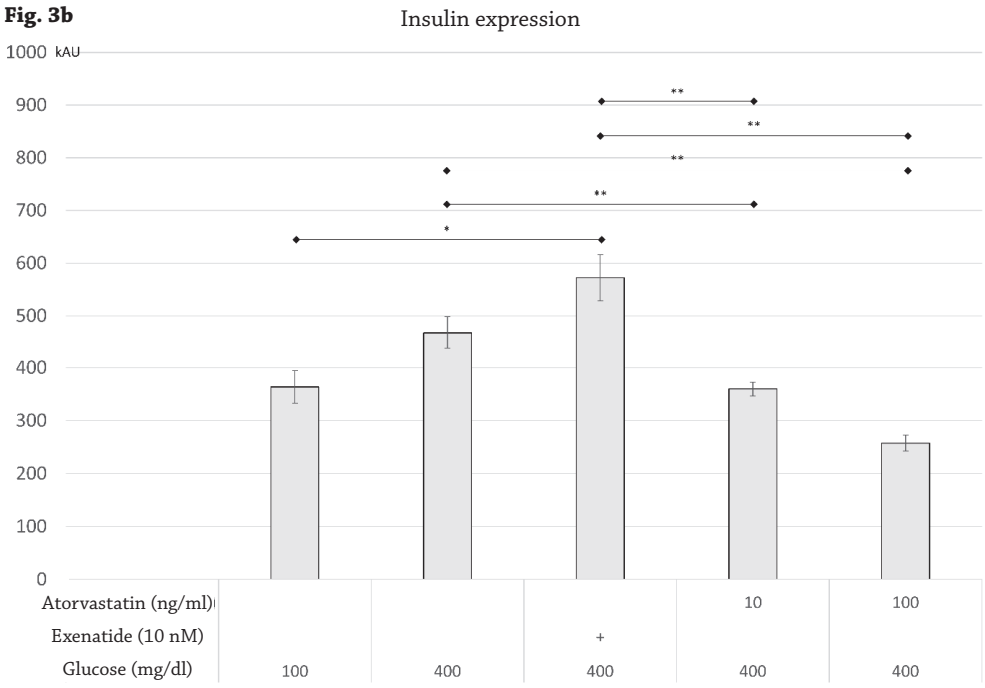


Fig. 3b


Insulin expression
1000 kAU
900
800
700
600
500
400
300
200
100
0
**
**
**
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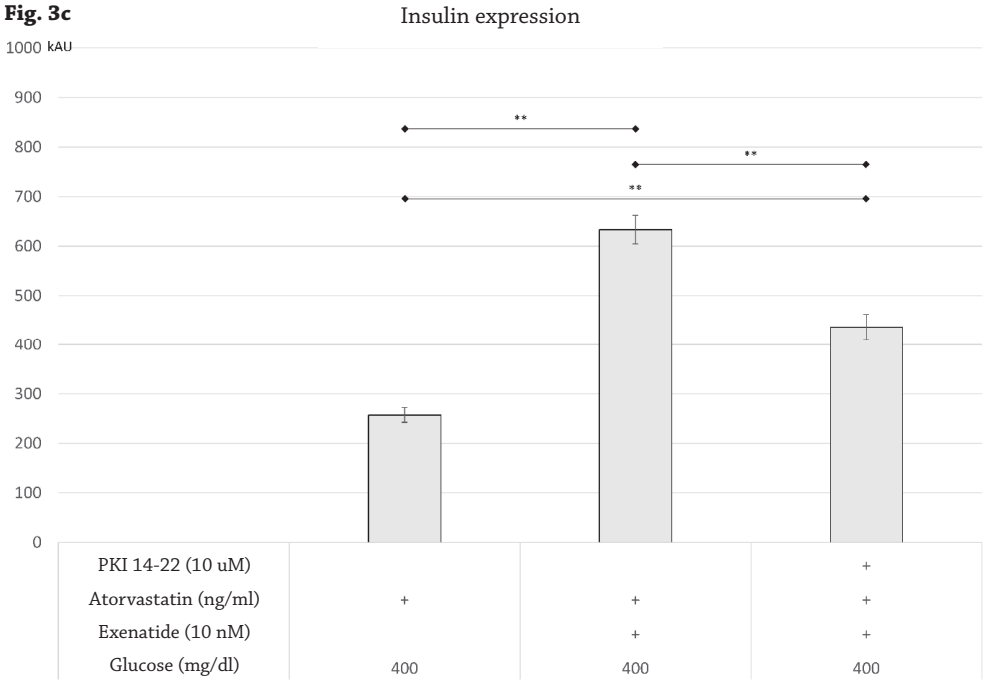
*
Atorvastatin (ng/ml) 10 100
Exenatide (10 nM) +
Glucose (mg/dl) 100 400 400 400 400

Fig. 3c


Insulin expression
1000 kAU
900
800
700
600
500
400
300
200
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0
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PKI 14-22 (10 uM) +
Atorvastatin (ng/ml) + + +
Exenatide (10 nM) + +
Glucose (mg/dl) 400 400 400

Fig. 3d

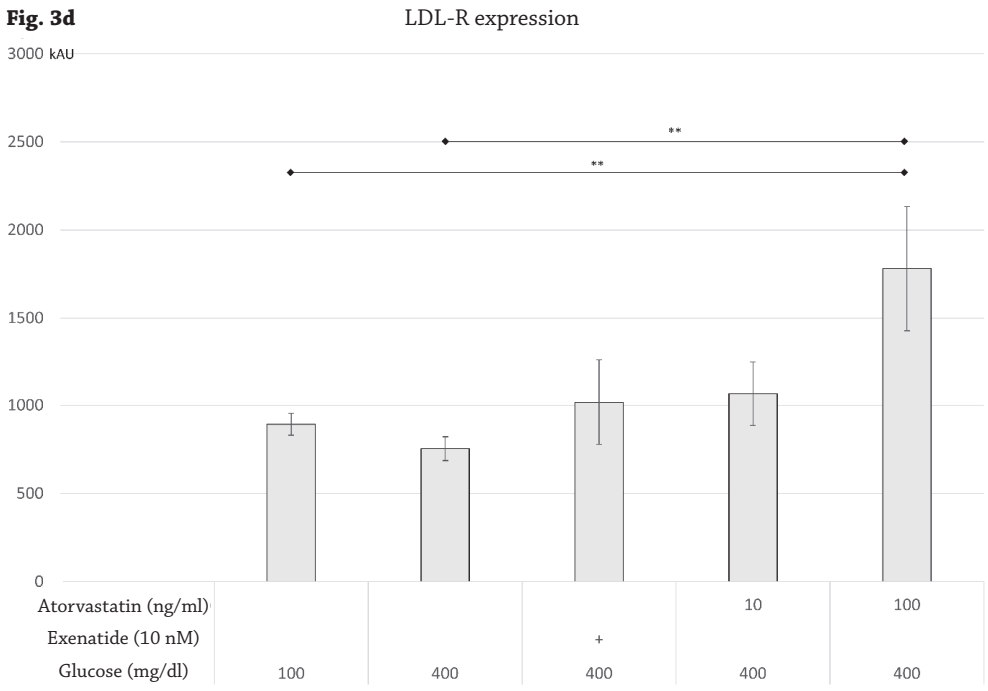

Fig. 3e

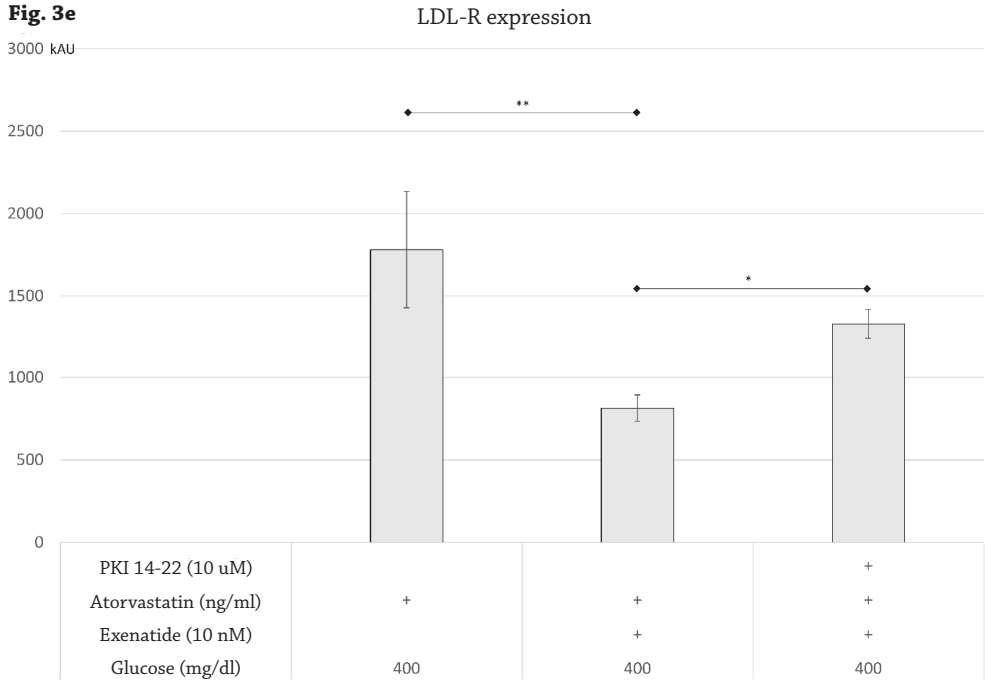

Fig. 3. Representative immunofluorescent images (a) showing the impact of atorvastatin and exenatide on the expression of insulin (**b–c**) and LDL-R (**d–e**). * <0.05; ** <0.01.

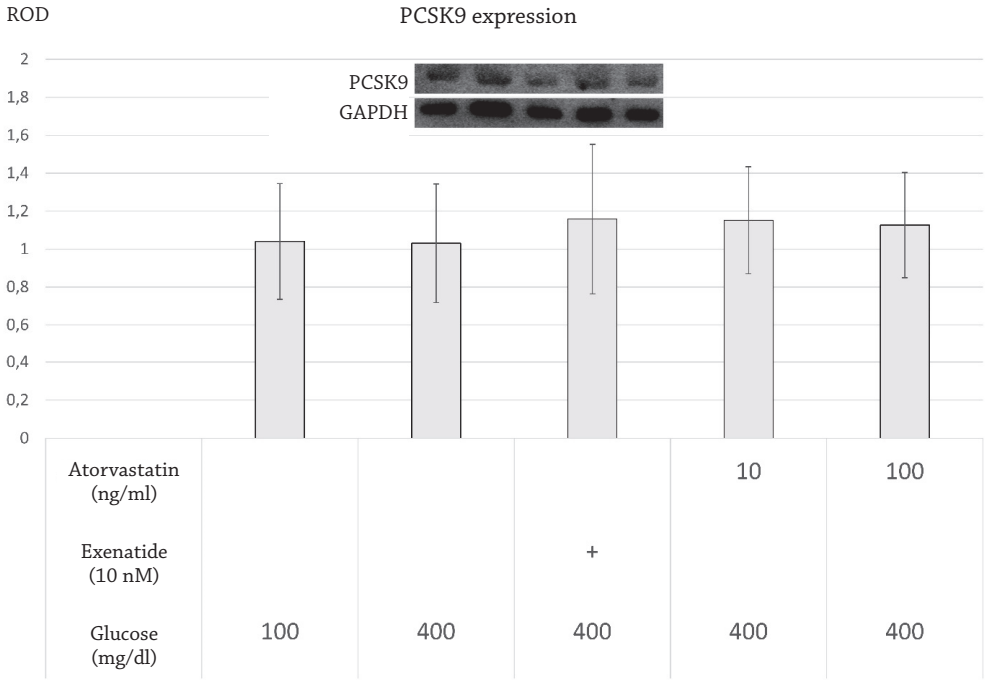

Fig. 4. The influence of atorvastatin and exenatide on PCSK9 expression

Discussion

Our experiments did not show a negative impact of statin on the viability of pancreatic beta cells. However, the insulin expression in hyperglycemic conditions was hampered by atorvastatin, both at mRNA and protein level. The concurrent rise in LDL-R expression in pancreatic islet beta cells exposed to atorvastatin might explain this observation – due to the connection with a high level of LDL-R, leading to increased cholesterol uptake and resulting in potential lipotoxicity. A dose dependency on the effects of atorvastatin on 1.1.E7 cells was also noted. Exenatide was able to reduce the impact of statin on beta cells in a PKA dependent pathway, showing improvements in insulin expression and reduction in LDL-R in cells exposed to atorvastatin. Furthermore, our experiments showed a decrease in the PCSK9 mRNA level in cells subjected to atorvastatin at high concentration. It seems that this impact might be mitigated by exenatide. Interestingly, no change in the protein expression of PCSK9 was noted in those experimental conditions. The discrepancy between the response at mRNA and protein level may result from temporal variations in gene triggering following a stimulus, as observed in previous studies (Golan-Lavi, 2017).

Data regarding slight diabetogenic effects of statins have been shown in several large scale clinical trials (Khan et al., 2019). Despite the increase in the number of new cases of diabetes and an increase in HbA1c level, statins – due to their beneficial role in atherosclerosis prevention – remain the drug of choice in the treatment of hyperlipidemias. Studies performed so far have not provided an unequivocal cause of worsened glucose metabolism. Some have suggested reduced insulin sensitivity (Grunwald et al., 2022), others reduced insulin level (Takei et al., 2020). In our study we explored the direct *in vitro* impact of atorvastatin on insulin expression, and showed that atorvastatin at both low and high concentration can attenuate insulin expression at protein level in human pancreatic islets beta cells subjected to atorvastatin. The influence on transcriptional activity was less evident and reached statistical significance in cells exposed to high atorvastatin concentration, which might reflect a potential influence on posttranslational modifications inflicted by statin exposure. Human *in vivo* studies give conflicting results on the subject, showing reduced insulin expression during atorvastatin therapy in patients with polycystic ovary syndrome (Chen and Zheng, 2021), or lack of negative impact on insulin concentration in hyperlipidemic subjects (Buldak et al., 2012). Whereas fluvastatin therapy was associated with increased insulin levels and increased insulin resistance in healthy volunteers (Felder et al., 2021). These inconsistencies may stem from different drugs used in the study design, showing better results under atorvastatin treatment and in different study populations. It seems that beneficial changes are seen in patient suffering from a disease that should be treated with statin (e.g., hyperlipidemia), but the influence on healthy volunteers may be absent or even harmful. Similar to our results on the expression of insulin were noted in *in vitro* cultured rat insulinoma cell line INS-1, in which atorvastatin led to a significant drop in insulin levels. This observation was connected with reduced activity of p-CREB after the exposure of cells to atorvastatin. One of the essential activators of CREB is PKA, which is thought to be a major contributor of intracellular signal transduction after stimulation of GLP-1 receptors. In the further steps of our experiments, we showed that exenatide can block the impact of atorvastatin on insulin expression at mRNA and protein level in a PKA-dependent manner. This is concordant with experiments in mouse beta cell line MIN6 exposed to simvastatin (Yaluri et al., 2015), which showed that GLP-1 pathway activation was able to prevent the reduction in insulin secretion caused by simvastatin. Unfortunately, there

are no comparative data on the impact of concomitant use of statin and GLP-1 receptor agonists on human pancreatic beta cell lines.

Simultaneously, with the impact on insulin expression, we explored the effects of atorvastatin on LDL receptor and PCSK9 expression. Both these proteins are associated with lipid metabolism. Increased LDL cholesterol uptake, which is responsible for lipid lowering effects, takes place predominantly in the liver (Hafner et al., 2011). However, increased cholesterol uptake was also observed in other organs and tissues (Sarsenbayeva et al., 2021). In this scenario, it was connected with abnormal organ function, leading to insulin resistance. Our results indicated that a high concentration of atorvastatin increased the expression of LDL receptors in pancreatic beta cells, which may lead to increased cholesterol uptake. Lipid accumulation in islet beta cells is thought to be responsible for cellular toxicity (Yang et al., 2020) and restrictions in dietary cholesterol were shown to improve beta cell function (Tricò et al., 2018). However, others have reported that atorvastatin treatment of hyperlipidemic mice resulted in a pancreatic reduction of LDL-R, which was accompanied by an increase in PCSK9 level (Yu et al., 2018). The reason for this divergence may be the method of acquisition of pancreatic tissue. In the above-mentioned experiment, the authors obtained whole pancreatic tissue sample, whereas in our experiment we relied on *in vitro* pure cell line. Protein expression varies among cellular populations. Such differences have been observed even among subpopulations of pancreatic islets cells. Therefore, the result might not show the precise impact of statin on pancreatic islet beta cells. As mentioned earlier, our data show that GLP-1 analog can increase insulin secretion in cells subjected to statin in culture media, but is also able to prevent the increase in LDL-R in culture conditions. This effect is predominantly mitigated by PKA, which was shown in experiments with PKA inhibitor. Available data from *in vivo* studies performed so far have not shown significant pharmacokinetic interactions between statins and GLP-1 analogs (Hausner et al., 2017). Therefore, one may assume that the observed impact is most probably based on pharmacodynamic level, which might be considered a viable therapeutic option in metabolic diseases associated with cellular lipid accumulation (e.g., non-alcoholic steatohepatitis) (Kothari et al., 2019).

Unlike low concentration, a high concentration of atorvastatin led to a reduction in PCSK9 mRNA expression. Exenatide completely blocked the effects of atorvastatin. Interestingly, in our experiments we did not find any statistically significant influence on PCSK9 protein expression in all experimental settings. These findings may reflect the different temporal response to stimulus between PCSK9 level. PCSK9 protein synthesis might take place later than LDL-R and potentiate the increase in LDL-R. Here, in the selected time frame, we might caught a temporary reduction in mRNA expression, which at that point have not yet been followed by protein expression. Previously, it has been noted that PCSK9 level is rising in parallel to increased LDL receptors levels (Dong et al., 2010), but it was also noted that mRNA response level was dependent on the statin and its concentration, and was negligible for atorvastatin at the highest concentrations for at 24 h (Blanchet et al., 2016). Liraglutide treatment in patients with DM2 resulted in a decrease in serum PCSK9, but only in those patients who achieved improvements in glucose control and without background statin therapy 214.9 ± 56.4 vs. 244.5 ± 99.2 ng/ml, $P = 0.024$) (Vergès et al., 2022). In patients receiving statin treatment, the PCSK9 was not affected by liraglutide (301.1 ± 91.5 vs. 281.2 ± 96.9 ng/ml, $P = 0.41$). Those results show that the regulation of PCSK9 expression is more complex than simple refractory response to increased LDL-R level. It is also worth mentioning that PCSK9 is expressed to a lesser extent in islet beta cells than in liver cells (Yu et al., 2018). Such a difference may be responsible for the different results between studies *in vitro* (e.g., pure pancreatic beta cell cultures without hepatocytes) and *in vivo* that are based on a whole living organism. A direct impact of liraglutide (a GLP-1 analog) on the reduction of PCSK9 expression was noted in hepatocytes cultures *in vitro* (Vergès et al., 2021). Another explanation for the minute impact on PCSK9 protein in our culture condition (in contrast to *in vivo* studies) is that the majority of PCSK9 in the pancreas seems to be expressed by islet delta cells (Da Dalt et al., 2019).

In summary, our experiments show that atorvastatin reduces insulin and increases LDL-R expression in cultured human pancreatic islets beta cells (1.1E7). These effects are mitigated by exenatide. The results support the hypothesis that an increased propensity to diabetes during statin therapy is connected with diminished insulin level and shows the promising role of combined therapy of metabolic diseases with GLP-1 receptor agonists.

Limitations of the current study should be kept in mind. Those include the *in vitro* setting, which cannot show the complexity of interactions in the whole organism. However, the employment of such a setting provides an opportunity to explore direct actions of drugs, whereas prolonged *in vivo* experiments (due to complex interactions) may overshadow the net effect of therapy.

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Ethical aspects and conflict of interests
The authors have no conflict of interests to declare.

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