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Interactions between Atorvastatin and the Farnesoid X Receptor Impair Insulinotropic Effects of Bile Acids and Modulate Diabetogenic Risk

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Interactions between Atorvastatin and FXR in Beta Cells

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Abbreviations: CDC: chenodeoxycholic acid, FXR: farnesoid X receptor, HMG-CoA: hydroxy-methyl-glutaryl coenzyme A, PXR: pregnane X receptor
Abstract:

Bile acids such as chenodeoxycholic acid (CDC) acutely enhance insulin secretion via the farnesoid X receptor (FXR). Statins, which are frequently prescribed for type 2 diabetic patients suffering from dyslipidemia, are known for their diabetogenic risk and are reported to interact with the FXR. Our study investigates whether this interaction is relevant for beta cell signaling and plays a role for negative effects of statins on glycemic control. Experiments were performed with islets and islet cells from C57BL/6N wildtype and FXR knock-out mice. Culturing islets with atorvastatin (15 µM) for 24 h decreased glucose-stimulated insulin secretion by approximately 30 % without affecting ATP synthesis. Prolonged exposure for 7 d lowered the concentration necessary for impairment of insulin release to 150 nM. After 24-h culture with atorvastatin, the ability of CDC (500 nM) to elevate $[\text{Ca}^{2+}]_c$ was diminished and the potentiating effect on insulin secretion was completely lost. Mevalonate largely reduced the negative effect of atorvastatin. Nuclear activity of FXR was reduced by atorvastatin in a mouse FXR reporter assay. The atorvastatin-induced decrease in insulin release was also present in FXR-KO mice. Though not a prerequisite, FXR seems to influence the degree of damage caused by atorvastatin in dependence of its interaction with CDC: Preparations responding to CDC with an increase in insulin secretion under control conditions were less impaired by atorvastatin than preparations that were non-responsive to CDC. Extended stimulation of FXR by the synthetic agonist GW4064, which is suggested to induce translocation of FXR from the cytosol into the nucleus, increased the inhibitory effect of atorvastatin. In conclusion, atorvastatin inhibits insulin release and prevents positive effects of bile acids on beta cell function. Both interactions may contribute to progression of type 2 diabetes mellitus.
Significance Statement

This study shows that the diabetogenic risk of statins is coupled to the activity of FXR-dependent signaling pathways in beta cells. On the one hand, statins abolish the insulinotropic effects of bile acids and on the other hand, FXR determines the level of impairment of islet function by the statin.
1. Introduction

Statins as LDL-cholesterol lowering drugs are in broad clinical use. Their beneficial effects concerning prevention of cardiovascular risk are undisputed (Scandinavian Simvastatin Survival Study study group, 1994; Sever et al., 2003), nevertheless certain side effects have to be considered (Thompson et al., 2016). A meta-analysis of 13 statin trials with 91140 participants provides clear evidence that the risk to develop type 2 diabetes mellitus increases in patients during long-term statin therapy (Sattar et al., 2010).

Particularly, lipophilic statins such as atorvastatin are assumed to influence islet function in a detrimental way (Yada et al., 1999; Yaluri et al., 2015; Urbano et al., 2017). Whether this is associated with the hydroxy-methyl-glutaryl coenzyme A (HMG-CoA) reductase inhibition or results from off-target effects, remains controversial. Partly the effects are closely linked to the mode of action of statins (Urbano et al., 2017), partly the observed effects are not reversible by a co-culture with mevalonate (Yaluri et al., 2015). Interestingly, it was shown that statins interact with pathways regulated by the farnesoid X receptor (FXR). Fu et al. reported effects of atorvastatin on FXR-induced target genes in mice (Fu et al., 2014), while Habeos et al. observed changes in the expression and DNA-binding activity of the receptor in the liver after treatment of syrian hamsters with simvastatin as well as in simvastatin-treated HepG2 cells (Habeos et al., 2005).

The FXR is a nuclear receptor targeted by bile acids like chenodeoxycholic acid (CDC), which is the most effective one (Makishima et al., 1999; Parks et al., 1999). It plays a role for regulation of lipid and bile acid metabolism, but also for glucose homeostasis (Fiorucci et al., 2009; Düfer et al., 2012a). FXR expression is high in liver, adrenal
glands and intestine (Huber *et al.*, 2002), however mRNA can also be found in other organs like the endocrine pancreas (Popescu *et al.*, 2010). In our previous work we investigated the impact of an acute stimulation of the FXR by the bile acid taurochenodeoxycholic acid (TCDC) on insulin secretion. We detected an FXR-dependent insulinotropic effect of the bile acid, which includes inhibition of $K_{ATP}$ channels, membrane depolarization and increased $Ca^{2+}$ influx (Düfer *et al.*, 2012b). For this acute stimulatory effect on insulin secretion, cytosolic localization of FXR and bile acid-induced interaction with $K_{ATP}$ channels are essential.

The reported impact of statins on FXR in liver and intestine, respectively (Habeos *et al.*, 2005; Fu *et al.*, 2014), raises the question of a comparable situation in the pancreas. The aim of our study was to gain insight into possible interactions between FXR and statins in pancreatic islets. Therefore, the influence of atorvastatin and its interaction with bile acid signaling was investigated in islets of wildtype and FXR knock-out (FXR-KO) mice by monitoring membrane potential, cytosolic $Ca^{2+}$ concentration ([Ca$^{2+}]_c$), ATP, apoptosis and insulin release and content. In addition, a reporter assay was used to test for interactions of statins with transcriptional activity of FXR.
2. Materials and Methods

2.1. Cell and Islet Preparation

Experiments were performed with islets of Langerhans from adult male and female C57BL/6N mice (Charles River, Sulzfeld, Germany) or adult male and female FXR-deficient mice (age of 9±3 months) from a C57BL/6N background described earlier (Sinal et al., 2000). The principles of laboratory animal care were followed according to German laws (Az. 53.5.32.7.1/MS-12668, health and veterinary office Münster, Germany). Mice were euthanized by CO₂. Islets were isolated by collagenase digestion. Dispersed cells or smaller cell clusters were obtained by trypsinization and used for membrane potential and [Ca²⁺]₅ measurements and for determination of apoptosis. Islets and cells were cultured in RPMI 1640 medium (11.1 mM glucose) supplemented with 10 % fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in 5 % CO₂ humidified atmosphere. After preparation islets or dispersed islet cells were kept overnight in standard culture medium. Next day, incubation started in the presence of atorvastatin or pravastatin for 24 h or 7 days. In case of pre-incubations with GW4064 atorvastatin was added one day later. Medium was changed every second or third day during the long-term incubation of 7 days.

2.2. Solution and Chemicals

Insulin secretion was performed in bath solution containing [mM]: 122 NaCl, 4.7 KCl, 1.1 MgCl₂, 2.5 CaCl₂, 10 HEPES (pH 7.4) and 0.5 % bovine serum albumin. [Ca²⁺]₅, membrane potential and ATP were measured in a solution which contained [mM]: 140 NaCl, 5 KCl, 1.2 MgCl₂, 2.5 CaCl₂, 10 HEPES (pH 7.4). Pipette solution for electrophysiology contained [mM]: 10 KCl, 10 NaCl, 70 K₂SO₄, 4 MgCl₂, 2 CaCl₂, 10
EGTA, 20 HEPES, 250 µg/ml amphotericin B, pH 7.15. Glucose was added as indicated.

Collagenase P was from Roche Diagnostics (Mannheim, Germany); annexin V reagent was from Essen BioScience (Michigan, USA); RPMI 1640, fetal calf serum, and penicillin/streptomycin were obtained from Life Technologies (Darmstadt, Germany). The statins used were: atorvastatin (IUPAC name: (3R,5R)-7-[2-(4-fluorophenyl)-3-phenyl-4-(phenylcarbamoyl)-5-propan-2-ylpyrrol-1-yl]-3,5-dihydroxyheptanoic acid, calcium trihydrate, purity: ≥ 98 %) and pravastatin (IUPAC name: (3R,5R)-7-[(1S,2S,6S,8S,8aR)-6-hydroxy-2-methyl-8-[(2S)-2-methylbutanoyl]oxy-1,2,6,7,8,8a-hexahydonaphthalen-1-yl]-3,5-dihydroxyheptanoic acid, sodium hydrate, purity: ≥ 98 %). Both statins, as well as GW4064 (IUPAC name: 3-[(E)-2-[2-chloro-4-[[3-(2,6-dichlorophenyl)-5-propan-2-yl]-1,2-oxazol-4-yl][methoxy][phenyl]ethenyl]benzoic acid) and pregnenolone 16alpha-carbonitrile (IUPAC name: (3S,8S,9S,10R,13S,14S,16R,17S)-17-acetyl-3-hydroxy-10,13-dimethyl-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthrene-16-carbonitrile) were from Sigma-Aldrich (Taufkirchen, Germany). Fura-2 AM was ordered from Biotrend (Köln, Germany). Labelled ¹²⁵I-insulin was kindly provided by Sanofi (Frankfurt, Germany), primary and secondary antibodies were from Merck Millipore (Rat Insulin RIA, RI-13K) provided by Biotrend (Köln, Germany). All other chemicals were from Sigma–Aldrich (Taufkirchen, Germany) or Diagonal (Münster, Germany).

2.3. Insulin Secretion and Content

Islets were moved to bath solution containing 5.6 mM glucose for 2 h after a culture period of 22 h or 7 days in the presence or absence of atorvastatin or pravastatin. During these 2 h the statin was still present. Following this, glucose was lowered to 3
mM for 1 h and bath solution did not contain atorvastatin or pravastatin anymore. Batches of five islets were incubated for 1 h at 37 °C with glucose concentrations and substances as described in the respective experiment. Thereafter, supernatant was removed for quantitative analysis and islets were lysed with acid ethanol in order to determine insulin content. Insulin was quantified by radioimmunoassay using rat insulin as standard.

2.4. Annexin V Assay

The live-cell analysis system IncuCyte® (Essen BioScience) was used for measurement of apoptosis. By means of this method cell viability could be monitored over a longer time period while cells were in stable conditions in an incubator. After preparation, dispersed islet cells were cultured in a 96 well plate overnight. The following day, cells were incubated in the presence or absence of 15 µM atorvastatin. 50 µM H2O2 was used as a positive control. After adding annexin V green reagent to each well as instructed by the manufacturer, the measurement started for a time period of 44 h (37 °C, 5 % CO2 humidified atmosphere). Changes in fluorescence of annexin V green reagent were monitored by excitation at 480 nm every 2 hours.

2.5. Determination of [Ca2+]c

Cells and cell clusters were loaded with the fluorescent dye fura-2-acetoxymethylester (fura-2 AM, 5 µM, 37 °C, 30 min). [Ca2+]c was determined by measuring fluorescence at 515 nm after alternate excitation with 340 and 380 nm, respectively. Cells were perfused with bath solution containing glucose and substances as indicated.
2.6. Electrophysiology

Changes in membrane potential of single islet cells were determined by the perforated-patch configuration of the patch-clamp technique in the current clamp mode (EPC-10 amplifier, HEKA). Giga-seals were obtained at ~24-26 °C by patch pipettes pulled from borosilicate glass. Cells were perifused with bath solution containing glucose and substances as indicated.

2.7. ATP Assay

For determination of ATP, 20 islets were incubated at a glucose concentration of 6 mM (0.1 % bovine serum albumin) for 1 h. Thereafter, the islets were treated with 0.5 or 15 mM glucose for 30 min (37 °C). Bath solution was quickly removed and islets were lysed by a solution containing NaOH (8 g/L) and EDTA-di-Na (0.186 g/L). ATP was determined according to the manufacturer’s protocol (ATP Determination Kit A22066, Invitrogen).

2.8. FXR Reporter Assay

Reporter cells (Indigo Bioscience Mouse Farnesoid X Receptor Reporter Assay™) expressing the luciferase gene under control of a promotor that is activated by FXR were thawed by addition of cell recovery medium provided by the manufacturer. 100 µl of the cell suspension were used per well. 100 µl of treatment medium supplemented with the respective test compounds (atorvastatin, GW4064, mevalonate or solvent) were added and incubated at 37 °C, 5 % CO₂ for 24 h. Next day, medium was removed, detection reagents were added and luminescence was measured (CLARIOstar®, BMG Labtech). Samples were run in duplicate (GW4064) or triplicate (all samples with atorvastatin pre-treatment) and were blank-corrected for evaluation.
2.9. Data Evaluation and Statistical Analysis

All experiments were performed with islets or cells from at least three independent mouse preparations. \([\text{Ca}^{2+}]_c\) was determined by calculating the area under the curve (AUC) after subtraction of background fluorescence and baseline correction 10 min before changes in bath solution (Figure 3C) or by determining mean \([\text{Ca}^{2+}]_c\) over a period of 10 min (Figure 2). Membrane potential was analyzed by averaging the values of the last 30 s before changes in bath solution. For quantification of apoptosis, the percentage of apoptotic area in relation to the whole cell area was determined. Values are presented as mean ± SD. To compare two single groups, Student’s t test was performed. For comparison among groups, statistical significance was assessed by analysis of variances (ANOVA) followed by Student-Newman-Keuls post-hoc test. The null hypothesis of each series of experiments was that the test compound has no influence on the respective parameter. Values of \(p \leq 0.05\) were considered as statistically significant.
3. Results

3.1. Atorvastatin decreases glucose-stimulated insulin secretion

To investigate the effect of atorvastatin on insulin secretion, two incubation periods with different concentrations of the HMG-CoA reductase inhibitor were chosen. Culturing islets in the presence of 15 µM atorvastatin for 24 h significantly decreased insulin release stimulated by 15 mM glucose. Islets exposed to 1.5 µM atorvastatin for 24 h only showed a tendency to reduced insulin secretion in response to stimulation with 15 mM glucose (Figure 1A). The culture period in medium supplemented with various concentrations of atorvastatin was extended to 7 days to investigate the influence of time. This prolonged treatment potentiated the detrimental effect of 15 µM atorvastatin on glucose-stimulated insulin secretion (24 h: 3.3 ± 0.9 ng/(islet*h), n = 10, vs. 7 d: 1.3 ± 0.9 ng/(islet*h), n = 13, p ≤ 0.001). Moreover, already nanomolar concentrations of the statin (150 and 500 nM) showed a negative effect on insulin release after 7 days (Figure 1B). Since extension of the time period to 7 days led to stronger variations in secretion among the individual preparations, absolute values were calculated as percentage of control (15 mM glucose) in this series of experiments. To investigate whether the reduction of insulin release by atorvastatin is related to inhibition of HMG-CoA, mevalonate (500 µM) was added to the culture medium. Mevalonate completely prevented the effect of atorvastatin regarding the 24-h-culture period (Figure 1C) and partly protected the islets during the extended treatment for 7 d (Figure 1D).
3.2. Atorvastatin changes cytosolic \( \text{Ca}^{2+} \), but has no effect on cell viability or ATP synthesis

Since the rise in \([\text{Ca}^{2+}]_c\) is crucial for insulin secretion, it was tested, whether the inhibitory effect of atorvastatin is caused by changes in \([\text{Ca}^{2+}]_c\). Incubating islet cells with 15 µM atorvastatin for 24 h slightly altered the \( \text{Ca}^{2+} \) response to a glucose stimulus: After exposure to atorvastatin, the first increase in \([\text{Ca}^{2+}]_c\) in response to 15 mM glucose was approximately half a minute delayed compared to standard conditions (Figure 2A). Apart from the effect on response time, pre-treatment with atorvastatin reduced the mean concentration of \([\text{Ca}^{2+}]_c\) (Figure 2B).

Insulin content was not affected even after incubating pancreatic islets in standard culture medium (10 mM glucose) supplemented with atorvastatin for the long-term period of 7 days (Figure 2C). Corresponding to these results there was no induction of apoptosis by the exposure of islet cells to 15 µM atorvastatin in standard culture medium for up to 44 h compared to the control (% apoptotic area after 24 h: control 8.0 ± 1.5 % vs. 15 µM atorvastatin, 24 h, 8.9 ± 2.6 %, n = 3, n. s.) (Figure 2D). As atorvastatin was reported to depolarize mitochondria isolated from rat pancreata and to reduce ATP content of the insulin-secreting cell line INS1 (Urbano et al., 2017, Sadighara et al., 2017), we tested whether atorvastatin affects glucose-stimulated ATP generation in murine islets. ATP content of islets stimulated with 0.5 or 15 mM glucose after 24-h culture with atorvastatin (15 µM) was not different compared to controls (Figure 2E) excluding severe impairment of mitochondrial function by the drug.

3.3. Atorvastatin abolishes the insulinotropic effect of the bile acid CDC

In order to evaluate if atorvastatin influences the FXR or its signaling pathway in pancreatic islets as described for the liver (Fu et al., 2014), the acute effect of the bile
acid CDC on insulin secretion was tested after culturing islets under control conditions or in the presence of 15 µM atorvastatin for 24 h. Additionally, 200 µM pravastatin were tested under the same conditions.

The acute application of CDC in a concentration of 500 nM enhanced insulin secretion under standard conditions as described earlier (Düfer et al., 2012b). Interestingly, culturing islets with 15 µM atorvastatin for 24 h abolished the acute insulinotropic effect of CDC (Figure 3A). In this series of experiments CDC elevated insulin secretion from 3.6 ± 1.2 to 4.7 ± 1.7 ng/(islet*h), n = 13, p ≤ 0.05. After culture with 15 µM atorvastatin for 24 h, glucose-stimulated insulin release (1 h) was 2.7 ± 1.2 ng/(islet*h) in the absence and 2.5 ± 1.1 ng/(islet*h) in the presence of CDC (500 nM) (n = 13, n. s.). This was also fact for the lower concentration of 1.5 µM atorvastatin (insulin release in response to 15 mM glucose after 24-h culture with 1.5 µM atorvastatin: 4.1 ± 2.3 ng/(islet*h) vs. same conditions + CDC 500 nM: 4.6 ± 3.4 ng/(islet*h), n = 6, n. s.).

The same experiment was performed with pravastatin, which is more hydrophilic than atorvastatin. 24-h culture with 200 µM pravastatin also inhibited the stimulatory effect of CDC (Figure 3B), pointing to a class effect of statins (insulin release in response to 15 mM glucose after 24-h culture with pravastatin: 3.6 ± 1.5 ng/(islet*h) vs. same conditions + CDC 500 nM: 3.1 ± 1.4 ng/(islet*h), n = 12, n. s.).

Since the effect of CDC on insulin secretion depends on a rise in \([Ca^{2+}]_c\), this parameter was measured in islet cells and cell clusters which were exposed to 15 µM atorvastatin prior to the experiment for 24 h. \([Ca^{2+}]_c\) was determined by calculating the area under the curve (AUC) after baseline correction. In agreement with the acute effect on insulin secretion described above, the acute application of 500 nM CDC provoked an increase in \([Ca^{2+}]_c\) under control conditions. This was significantly diminished after exposure of the cells to atorvastatin (Figure 3C), explaining the ineffectiveness of CDC on insulin.
secretion after treatment with the lipophilic statin. As the effect of bile acids on \([\text{Ca}^{2+}]_c\) is regulated by electrical activity, a drug eliminating the CDC-induced changes in \([\text{Ca}^{2+}]_c\) is expected to interact with the influence of CDC on membrane potential. To verify this hypothesis, membrane potential of beta cells was determined in the perforated-patch configuration. These experiments were started in the presence of 5.5 mM glucose, i.e. a concentration in the range of the threshold for induction of \(\text{Ca}^{2+}\) action potentials. CDC was added at a concentration of 500 nM for approximately 7 min. If the seal was stable enough, glucose was elevated to 15 mM (CDC still present) at the end of the experiment to verify metabolic integrity. Application of CDC depolarized the membrane in control cells but did not induce any change in cells pretreated with 15 µM atorvastatin for 24 h (Figure 3D). Of note, most of the atorvastatin-pretreated cells appeared to be more depolarized at 5.5 mM glucose compared to the control cells. The lack of effect of CDC after application of the statin supports the idea that these drugs disrupt the coupling of the CDC/FXR-pathway and electrical activity, thereby reducing the effect of CDC on \(\text{Ca}^{2+}\) influx.

3.4. Atorvastatin interacts with FXR-response elements

To test whether atorvastatin influences the activity of FXR, a mouse luciferase reporter assay was used. Genetically manipulated cells, where expression of luciferase is controlled by an FXR-responsive promoter, were cultured either with 15 µM atorvastatin alone or in combination with 1 µM of the synthetic FXR activator GW4064 for 24 h and luciferase-catalyzed changes in bioluminescence were monitored thereafter. While atorvastatin did not influence FXR activity per se, GW4064-induced activation was dose-dependently diminished (Figure 4A and B). Interestingly,
additional treatment with mevalonate (500 µM) during culture partly protected against the inhibition of FXR by atorvastatin (Figure 4C).

As 10 µM atorvastatin were reported to activate the CYP-regulating nuclear pregnane X receptor (PXR) (Howe et al., 2011), we also checked whether activation of this receptor might mimic the negative influence of atorvastatin on insulin release. Therefore, the PXR agonist pregnenolone-16α-carbonitrile was used. 10 or 25 µM of this compound only showed a slight, non-significant tendency to reduce glucose-stimulated insulin release (Figure 4D) and did not mirror the effect of atorvastatin.

3.5. Inhibitory effect of atorvastatin in FXR-deficient islets

We demonstrated in previous work that the increase in [Ca\(^{2+}\)]\(_{c}\) in response to bile acids depends on FXR and is absent in beta cells of FXR-KO mice (Düfer et al., 2012b). Consequently, the results described above indicate an interaction between statins and the FXR leading to elimination of the insulinotropic effect of CDC. This interaction provoked the question whether there might also be an impact vice versa. Therefore, the experiments shown in figure 1A were repeated with pancreatic islets from FXR-deficient mice. After exposing FXR-KO islets to different concentrations of atorvastatin for 24 h, 15 µM atorvastatin caused a significant decline in insulin secretion, while a concentration of 1.5 µM – similar to the effect in wildtype islets – only tended to reduce insulin release (Figure 4E).

Comparison of the amounts of insulin, secreted in response to 15 mM glucose (1 h, expressed as percentage of control) after 24-h culture of the islets with 15 µM atorvastatin, revealed no difference between wildtype and FXR-deficient islets (insulin secretion after 24-h culture with atorvastatin (15 µM): wildtype 64.7 ± 28.9 % of control, n = 27, vs. FXR-KO 66.6 ± 29.6 % of control, n = 8, n. s.).
3.6. FXR modulates the damaging effect of atorvastatin

Although the experiments with FXR-deficient islets show the independency of the negative effect of atorvastatin on insulin secretion from the nuclear receptor per se, we made a remarkable observation after detailed analysis of the experiments with wildtype islets. Of 27 experiments with wildtype islets not every preparation responded to the acute application of 500 nM CDC with an increase in insulin secretion under control conditions. Some preparations were non-responsive to the bile acid, which means that the cytosolic $K_{ATP}/Ca^{2+}$-dependent signaling pathway of FXR is not operative. This might indicate a shift in the localization of FXR from the cytosol to the nucleus of the cell (Schittenhelm et al., 2015). To address this issue, the preparations were divided into two groups, CDC-responsive and CDC-non-responsive experiments, for subgroup analysis. Thereby, we detected a modulatory role of the FXR concerning the degree of damage caused by atorvastatin. Atorvastatin seems to be less harmful, when islets are CDC-responsive, while the inhibitory effect is aggravated, when islets are non-responsive to CDC under control conditions (Figure 5A): After 24-h treatment with 15 µM atorvastatin, glucose-stimulated insulin secretion was reduced to $77.0 \pm 28.4 \%$ of control in CDC-responsive preparations ($n = 17$). By contrast, 24-h treatment of CDC-non-responsive preparations with atorvastatin lowered glucose-stimulated insulin release to $43.8 \pm 14.3 \%$ of control ($n = 10$). Noteworthy, we observed that the inhibitory effect of atorvastatin on insulin release of CDC-non-responsive wildtype preparations was higher than in FXR-KO islets (Figure 5B). This points to a more negative effect of statins when FXR is still present but lost its influence on the cytosolic pathway compared to complete absence of the receptor.

To confirm that FXR influences the damaging effect of atorvastatin, experiments were performed in which the FXR was forced into the nucleus of the cell. To achieve this,
islet cells were exposed to the strong, synthetic FXR agonist GW4064 for an extended period. Previous work showed that the cytosolic, K\textsubscript{ATP}-dependent signaling pathway of FXR is inactive after prolonged receptor activation by GW4064 (Schittenhelm \textit{et al.}, 2015). To be able to compare the degree of damage caused by atorvastatin in dependence of the ability of FXR to act as a cytosolic receptor, only preparations responding to 500 nM CDC under control conditions, but not after 48-h culture with GW4064 were included. Figure 5C shows that GW4064-treated islets, which have lost the cytosolic pathway of FXR (dark grey vs. light grey bar), were much more sensitive to the inhibitory influence of atorvastatin compared to control (black vs. white bar). As already described above, atorvastatin had only a small effect on islets of CDC-responsive preparations (~ 14 % inhibition in this series of experiments). After pre-incubation of the islets with GW4064 (500 nM) for 24 h and addition of the statin for another 24 h, insulin secretion induced by 15 mM glucose was diminished by 80 %. Of note, glucose-stimulated insulin release was ~ 2-fold higher after 48-h culture with GW4064 compared to control.

Finally, the influence of HMG-CoA reductase on the interaction between atorvastatin and CDC/FXR was elucidated. Therefore, mevalonate (500 µM) was added to the culture medium in addition to atorvastatin. As expected, CDC did not stimulate insulin release after treatment with atorvastatin for 24 h. However, stimulation of islets with 500 nM CDC was clearly improved in those islets where inhibition of endogenous mevalonate synthesis was compensated by external mevalonate supplementation (Figure 5D).
4. Discussion

In accordance with other publications (Zhao and Zhao, 2015; Urbano et al., 2017) incubation of pancreatic islets with atorvastatin reduced insulin secretion. Noteworthy, experiments varied among species and concentration of atorvastatin. We tested atorvastatin in concentrations up to 15 μM. The high concentration, which exceeds circulating plasma levels in patients, was used to mimic accumulation of the lipophilic drug in vitro within a relatively short period of time, as isolated beta cells are difficult to culture for several weeks. To test, whether lower concentrations are also effective, we extended our protocol to 7 days. We observed that the effect of atorvastatin is not only dose-dependent (Figure 1A), but also time-dependent: Besides the potentiation of the detrimental effect of 15 μM atorvastatin, a decrease in insulin release was already provoked by nanomolar concentrations (150 and 500 nM) of the statin after long-term treatment for 7 d (Figure 1B). This concentration range is still approximately 3- to 4-fold higher than plasma concentrations reported in bioequivalence studies (e. g., Public assessment report, UK/H/3430/004/DC), but one must keep in mind that in vitro the drug has to cross the barrier of connective tissue surrounding murine islets and is not distributed via systemic circulation.

The inhibitory effect of atorvastatin was not accompanied by a decrease in glucose-stimulated ATP synthesis but involves changes in Ca^{2+} influx (Figures 2A, B and E) which points to an interaction with the triggering pathway that regulates beta cell function. Even though the exact mechanism remains to be elucidated, the diabetogenic effect of atorvastatin is unquestioned. Insulin content was not altered between control and any of the applied concentrations of atorvastatin even after 7 d (Figure 2C). However, glucose-stimulated insulin release could be fully restored when mevalonate
was added to the islets during the 24-h culture with atorvastatin. This suggests, that downstream metabolites of the cholesterol biosynthesis pathway are involved. The mevalonate rescue was only partial in the long-term experiments of 7 d, indicating that the deleterious effect of atorvastatin does not completely depend on inhibition of HMG-CoA reductase. As some statins activate the xenobiotic receptor PXR (Howe et al., 2011), we tested whether the known PXR agonist pregnenolone carbonitrile induces inhibitory effects similar to atorvastatin. The lack of any significant effect after 24-h culture (Figure 4D) argues against a major role of such an interaction in beta cells.

In contrast to reports of others (Zhao and Zhao, 2015; Sadighara et al., 2017), we could not attribute the detrimental effect on insulin secretion to changes in cell viability. These discrepancies might result from different experimental approaches, as the effect of atorvastatin on cytochrome c release was monitored in isolated mitochondria (Sadighara et al., 2017) and cell survival was investigated by an MTT assay which does not give any information about apoptosis (Zhao and Zhao, 2015). In summary, an effect of atorvastatin on insulin biosynthesis or a dramatic loss of beta cell mass can be ruled out as explanations for the decrease in insulin release in our investigation. Our data show for the first time that statins interfere with the insulinotropic effect of bile acids. In the postprandial state plasma levels of bile acids can rise up to 15 µM (Everson, 1987; Houten et al., 2006). This is not only necessary for the absorption of lipids, but also important for lowering blood sugar peaks by stimulation of insulin secretion. The stimulatory effect of the FXR-agonistic bile acid CDC, we already described in our previous investigations (Düfer et al., 2012b; Schittenhelm et al., 2015), was clearly seen under control conditions, but completely disappeared after 24-h culture with atorvastatin or pravastatin (Figure 3A and B). Corresponding to this loss
of efficacy on glucose-stimulated insulin release, the influence of CDC on membrane potential was impaired and the CDC-mediated increase in \([\text{Ca}^{2+}]_c\) was reduced to less than 50 % (Figure 3C and D). For patients being treated with statins, this implies not only a decline in islet function induced by the statin per se, but also the loss of an important physiological regulatory function of bile acids for glucose homeostasis. In addition, a reduction of the postprandial plasma concentrations of bile acids was reported in obese (Glicksman et al., 2010) and pre-diabetic (Shaham et al., 2008) patients, actually patient groups with the common indication for cholesterol-lowering drugs such as statins. Taken together, the already reduced contribution of bile acids to the postprandial regulation of blood glucose concentration in those patients would be progressively more or, worst case, entirely abolished during long-term therapy with statins. The exact mechanism leading to inhibition of the effect of CDC needs to be further investigated. Since the link between FXR activation and \(K_{\text{ATP}}\) channel closure has not yet been clearly identified, it might be possible that incubation with atorvastatin interferes with some target downstream to FXR, but upstream to the closure of \(K_{\text{ATP}}\) channels. Bearing in mind that statins impair the prenylation and thereby regulation of proteins by inhibiting the mevalonate pathway (Li et al., 1993), atorvastatin could disrupt some factor derived from this pathway linking FXR stimulation to \(K_{\text{ATP}}\) channel closure. In agreement with this assumption, co-culture with mevalonate rescued the sensitivity of islets to acute stimulation with CDC (Figure 5D).

Furthermore, our results demonstrate that the interference between atorvastatin and the FXR is not unilateral, but of a bidirectional character. The experiments with FXR-KO islets show that the impairing effect induced by atorvastatin is partly independent of FXR (Figure 4E). But in addition, our data reveal that FXR plays a modulatory role
with regard to the degree of damage caused by the statin (Figure 5A and B). The availability of the receptor in the cytosol seems to be crucial for this modulation. Apparently, a localization close to the plasma membrane that enables interaction of FXR with the cytosolic triggering pathway for insulin secretion via $\text{K}_{\text{ATP}}$ channel closure and $\text{Ca}^{2+}$ influx is protective and reduces the negative effect of atorvastatin on insulin release. Disruption of this pathway is associated with an increased inhibitory effect of statins on insulin release (Figure 5C).

Popescu et al. detected a translocation of the FXR into the nucleus in obese $\text{ob/ob}$ mice, whereas the receptor is mainly located in the cytosol in wildtype beta cells (Popescu et al., 2010). Howe et al. (2011) reported that atorvastatin was not able to activate the FXR. We confirmed this result in a mouse FXR reporter assay. Furthermore, we demonstrate for the first time that atorvastatin inhibits nuclear FXR activity (Figure 4B). If this also applies to pancreatic beta cells, suppression of genes important for insulin secretion might explain the increased level of beta cell failure under conditions where the FXR is forced to translocate into the nucleus. Astonishingly, the inhibitory influence of atorvastatin on luciferase expression was reduced by mevalonate. This may further support the idea, that HMG-CoA reductase-dependent modifications of FXR are generally required for regular function of the receptor with respect to both, cytosolic and nuclear signaling. The nature of these alterations as well as the FXR-regulated target genes that are suppressed by atorvastatin have to be characterized in further studies.

With respect to the pathway described above, differences in receptor localization – and thereby in receptor function – between lean and obese organisms might be critical
determinants for the statin-bile acid interaction. In line with the idea of fundamental differences in FXR function, dependent on body weight and/or lipid homeostasis, it was reported that ablation of FXR is associated with decreased insulin secretion and insulin content under control conditions as well as peripheral insulin resistance (Cariou et al., 2006; Popescu et al., 2010). However, these negative characteristics completely change in a glucolipotoxic environment. FXR-deficient islets were resistant to glucolipotoxicity, while wildtype islets kept in glucolipotoxic medium showed an impaired glucose-stimulated insulin release (Schittenhelm et al., 2015). Supporting the hypothesis, that – in beta cells – cytosolic localization of FXR is a prerequisite for its positive impact on cellular function, Schittenhelm et al. demonstrated that the potentiating effect of FXR agonists on insulin secretion is lost in islets derived from mice after high-fat diet (Schittenhelm et al., 2015). Taken together, these data suggest that certain conditions (i.e. genetic or diet-induced obesity in vivo, glucolipotoxicity or prolonged receptor activation in vitro) disrupt the cytosolic interaction between FXR and KATP channels and might thereby aggravate the diabetogenic risk of statins. It is tempting to speculate that obese patients might be exceptionally sensitive to statin-induced beta cell damage as – similar to the genetic mouse model or to islets kept in a glucolipotoxic environment – their profile of FXR distribution and interaction may change during disease. Thereby, they not only lose the beneficial effect of bile acids on the endocrine pancreas, but also risk progression of beta cell failure by direct statin-induced impairment of glucose-stimulated insulin release.
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Authorship Contributions

Participated in research design: Hoffmeister, Drews, Düfer

Conducted experiments: Hoffmeister, Kaiser, Lüdtke

Performed data analysis: Hoffmeister, Kaiser, Lüdtke, Düfer

Wrote or contributed to the writing of the manuscript: Hoffmeister, Kaiser, Lüdtke, Drews, Düfer
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Footnotes

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Figure Legends

Figure 1

The influence of atorvastatin on insulin secretion depends on concentration and incubation time. A) Culturing islets of Langerhans for 24 h in standard medium supplemented with atorvastatin caused a decrease in glucose-stimulated insulin release (1 h steady-state incubation) in a concentration-dependent manner. B) Extending culture time to 7 d significantly increased the damaging effect of the statin. C, D) The effect was fully prevented (C) or partly reduced (D) by co-culture with mevalonate. The number of independent preparations is given below the bars of each diagram. Islets were isolated from female/male mice as follows: 4/6 (A), 6/9 (B), 3/9 (C), 0/6 (D). #, p ≤ 0.001 vs. all other conditions; §, p ≤ 0.05 vs. atorvastatin (D); *, p ≤ 0.05; **, p ≤ 0.01; ***, p ≤ 0.001.

Figure 2

The detrimental impact of atorvastatin on insulin secretion is associated with altered [Ca\(^{2+}\)]\(_{\text{c}}\), but not with changes in insulin content, apoptosis or intracellular ATP. A) After acutely stimulating islet cells with 15 mM glucose the initial increase in [Ca\(^{2+}\)]\(_{\text{c}}\) was delayed in those cells that were exposed to atorvastatin before (15 µM, 24 h). B) The increase in [Ca\(^{2+}\)]\(_{\text{c}}\) was reduced as well. C) No difference in insulin content was observed after culturing islets for 7 d in the presence or absence of atorvastatin (0.15 - 15 µM). D) Atorvastatin (15 µM) did not alter the fraction of apoptotic cell area vs. control. H\(_2\)O\(_2\) (50 µM) was included as a positive control in each experiment. E) ATP generation in response to 0.5 or 15 mM glucose was not impaired after 24 h application of atorvastatin. In (A) a representative recording is shown for each
condition. The diagram in (B) summarizes the mean \([Ca^{2+}]_c\) determined in the first 10 minutes after the rise in \([Ca^{2+}]_c\) induced by changing bath solution from 0.5 to 15 mM glucose. The number of cells (A, B) or independent preparations (C, E) is given below the bars of each diagram. Data presented in (D) are obtained from 3 independent experiments. Islets were isolated from female/male mice as follows: 0/3 (A, B), 6/3 (C), 3/0 (D), 1/2 (E). ***, p ≤ 0.001 vs. control (15 mM glucose); #, p ≤ 0.01 vs. 15 mM glucose + atorvastatin and 15 mM glucose, respectively (E).

**Figure 3**

The insulinotropic effect of the bile acid CDC is abolished after exposure to atorvastatin or pravastatin. A, B) The acute application of 500 nM CDC increased insulin secretion under stimulatory conditions (15 mM glucose, 1 h). After exposure to atorvastatin (15 µM) or pravastatin (200 µM) for 24 h the stimulatory effect of CDC was abolished. C) Under stimulatory conditions (15 mM glucose) CDC caused a rise in \([Ca^{2+}]_c\). Pre-treatment with atorvastatin (15 µM) for 24 h significantly reduced the bile acid-mediated increase in \([Ca^{2+}]_c\). D) Under control conditions, CDC induced electrical activity in beta cells in the presence of 5.5 mM glucose. This effect was abolished after pre-treatment with atorvastatin (15 µM, 24 h). In (C) and (D) representative recordings for each condition are shown, the diagram in (C) summarizes the percentage increase in \([Ca^{2+}]_c\) after application of CDC. \([Ca^{2+}]_c\) was determined by calculating the area under the curve (AUC) after baseline correction. The diagram in (D) illustrates the evaluation of the membrane potential before and after addition of CDC in control or atorvastatin-treated cells. The number of independent preparations (A, B) or cells (C, D) is given below the bars of each diagram. Islets were isolated from female/male mice.
as follows: 6/7 (A), 8/4 (B), 2/2 (C), 2/2 (D). #, p ≤ 0.001 vs. all other conditions; *, p ≤ 0.05; **, p ≤ 0.01; ***, p ≤ 0.001.

Figure 4

Nuclear activity of FXR is inhibited by atorvastatin but FXR is not essential for statin-mediated inhibition of insulin secretion. A) Reporter cells expressing luciferase under control of the FXR were treated with increasing concentrations of atorvastatin or GW4064 for 24 h. B, C) To run the assay in the antagonist mode, reporter cells were incubated with 1 µM of the synthetic FXR agonist GW4064 for 24 h. This was combined with different concentrations of atorvastatin without (B) or with (C) 500 µM mevalonate. Thereafter, substrate was added and luminescence was determined. Atorvastatin was without effect per se but inhibited the increase in luminescence induced by GW4064. This increase was partly outweighed by application of mevalonate. D) Pregnenolone carbonitrile (10 and 25 µM, 24 h) did not affect glucose-stimulated (15 mM glucose, 1 h) insulin secretion. E) Culturing pancreatic islets of FXR-deficient mice for 24 h in standard medium supplemented with atorvastatin (15 µM) decreased glucose-induced insulin secretion in a concentration-dependent manner similar to wildtype islets. The number of independent preparations is given below the bars of the diagram. Islets were isolated from female/male mice as follows: 2/4 (D), 4/4 (E). #, p ≤ 0.001 vs. all other conditions; *, p ≤ 0.05.

Figure 5

FXR modulates the negative effect of atorvastatin in dependence of the responsivity of the islets to CDC. A) The degree of inhibition caused by atorvastatin
(15 µM) was more pronounced in preparations that were non-responsive to 500 nM CDC (grey bar) under standard conditions (i.e. 1-h stimulation with 15 mM glucose) compared to those 17 out of 27 preparations that showed a potentiating effect in response to CDC (black bar). B) CDC-non-responsive islet preparations were more sensitive to statin-induced reduction of insulin release than FXR-KO islets. C) Islets were cultured with GW4064 (500 nM) for 48 h. After 24 h, atorvastatin (15 µM) was added for 24 h. For evaluation only those preparations were included in which GW4064 abolished the acute stimulatory effect of CDC. In these preparations atorvastatin only tended to decrease insulin release in response to 15 mM glucose (black vs. white bar). In islets exposed to GW4064 before and during atorvastatin treatment (dark grey bar) the inhibitory effect was drastically increased compared to both, GW4064-treated and control islets. D) Addition of mevalonate during the 24-h culture partly restored the responsivity of islets to acute stimulation with CDC. Dashed lines mark the groups chosen for statistic comparison. The number of independent preparations is given below the bars of each diagram. Islets were isolated from female/male mice as follows: 12/15 (A), 6/12 (B), 3/0 (C), 6/4 (D). #, p ≤ 0.001 (A, C, D) vs. all other conditions; §, p ≤ 0.05 vs. atorvastatin and GW4064; *, p ≤ 0.05; **, p ≤ 0.01; ***, p ≤ 0.001.
Figure 1

A

B

C

D

insulin secretion (ng/(islet*h))

insulin secretion (% of control)

insulin secretion (ng/(islet*h))

insulin secretion (% of control)

glucose (mM), 1 h atorvastatin (µM), 24-h pre-treatment

glucose (mM), 1 h atorvastatin (µM), 7-d pre-treatment

glucose (mM), 1 h atorvastatin (µM), mevalonate (µM), 24-h pre-treatment

glucose (mM), 1 h mevalonate (µM), 7-d pre-treatment

(10) 3 15 15 15

(15) 3 15 15 15 15 15 15 15

(12) 3 15 15 15 15

(12) 3 15 15 15 15

(6) - - - 15 15

(6) - - - 500 - 500

(6) - - - 500 - 500

(10) - - - 15 15

(10) - - - 500 - 500

(10) - - - 500 - 500

(10) - - - 500 - 500

(12) - - - 15 15

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(13) - - - 500 - 500

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(13) - - - 500 - 500

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Figure 2
**Figure 3**

### A

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### B

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### C

**control, 24 h**

- 15 mM glucose
- 0.5 mM glucose
- 500 nM CDC
- 15 mM glucose
- 0.5 mM glucose
- 500 nM CDC
**Figure 3**

**D**

- **control, 24 h**
  - 5.5 mM glucose 15 mM glucose
  - 500 nM CDC

- **atorvastatin, 24 h**
  - 5.5 mM glucose 15 mM glucose
  - 500 nM CDC

**Data:***

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**Legend:**
- *p < 0.05
**Figure 4**

**A**
- GW4064
- atorvastatin

**B**
1 µM GW4064

**C**
- atorvastatin + 500 µM MVL
- atorvastatin

**D**
- Insulin secretion (ng/islet*h)
- Glucose (mM), 1 h preg. carbonitrile (µM), 24-h pre-treatment
- (6) 3 15 15 15
- (6) 10 25
- (8) 3 15 15 15
- (8) 1.5 15

**E**
- FXR-KO
- Insulin secretion (ng/islet*h)
- Glucose (mM), 1 h atorvastatin (µM), 24-h pre-treatment
- (8) #
- (8) 3 15 15 15
- (8) 1.5 15
Figure 5

A

insulin secretion (% of control)

CDC-responsive preparations

+  -

atorvastatin (µM), 24-h pre-treatment glucose (mM), 1 h

glucose (mM), 1 h 15 15 15
atorvastatin (µM), 24-h pre-treatment

B

insulin secretion (% of control)

CDC-non-responsive FXR-KO

** *

atorvastatin (µM), 24-h pre-treatment glucose (mM), 1 h 15 15

C

insulin secretion (ng/islet*h))

#, §

atorvastatin (µM), 24-h pre-treatment glucose (mM), 1 h 3 15 15 15 15

D

insulin secretion (% of control)

atorvastatin (µM), 24-h pre-treatment glucose (mM), 1 h 3 15 15 15 15 15 15 15

CDC (nM), 1 h 500 500 500 500 500 500 500

atorvastatin (µM), 24-h pre-treatment mevalonate (µM), 24-h pre-treatment 500 500

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