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Increased uptake of oxLDL does not exert lipotoxic effects in insulin-secreting cells

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Abstract

Modified lipoproteins can negatively affect beta cell function and survival. However, the mechanisms behind interactions of modified lipoproteins with beta cells – and in particular, relationships to increased uptake – are only partly clarified. By over-expressing the scavenger receptor CD36 (Tet-on), we increased the uptake of fluorescent low-density modified lipoprotein (oxLDL) into insulin-secreting INS-1 cells. The magnitude of uptake followed the degree of CD36 over-expression. CD36 over-expression increased concomitant efflux of ^{3}H-cholesterol in proportion to the cellular contents of ^{3}H-cholesterol. Exposure to concentrations of oxLDL from 20 to 100 µg/mL dose-dependently increased toxicity (evaluated by MTT) as well as apoptosis. However, the increased uptake of oxLDL due to CD36 over-expression did not exert additive effects on oxLDL toxicity – neither on viability, nor on glucose-induced insulin release and cellular content. Reciprocally, blocking CD36 receptors by Sulfo-N-Succinimidyl Oleate decreased the uptake of oxLDL but did not diminish the toxicity. Pancreatic islets of CD36$^{-/-}$ mice displayed reduced uptake of ^{3}H-cholesterol-labeled oxLDL vs wild type but similar toxicity to oxLDL. OxLDL was found to increase the expression of CD36 in islets and INS-1 cells. In summary, given the experimental conditions, our results indicate that (1) increased uptake of oxLDL is not responsible for toxicity of oxLDL, (2) increased efflux of the cholesterol moiety of oxLDL counterbalances, at least in part, increased uptake and (3) oxLDL participates in the regulation of CD36 in pancreatic islets and in INS-1 cells.

Key Words

- cholesterol efflux
- lipoproteins
- insulin secretion
- islets of Langerhans
- doxycycline
- flow cytometry

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Introduction

Dyslipidemia in diabetes is characterized by elevated triglycerides, low concentrations of HDL and increased concentrations of small dense LDL-cholesterol particles, including oxidized LDL (oxLDL) (Mooradian 2009). A link between diabetic dyslipidemia and cardiovascular disease is well established. Emerging evidence indicates harmful influence of blood lipids on beta cell function, possibly aggravating the disease. Thus, oxLDL reduces beta cell capacity to release insulin by inhibition of preproinsulin mRNA (Okajima *et al.* 2005) and by promoting beta cell death (Abderrahmani *et al.* 2007). However, the ways

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by which oxLDL damages pancreatic beta cells is still largely unknown.

Beta cells have functional LDL receptors (Grupping *et al.* 1997), which bind native LDL (nLDL) but not oxLDL. Different scavenger receptors have been implicated in the binding and internalization of oxLDL (Moore & Freeman 2006). Evidence from tissues such as muscle, liver and macrophages suggests that the scavenger receptor CD36 plays a central role in the uptake of oxLDL (Collot-Teixeira *et al.* 2007). CD36 is essential for oxLDL uptake in macrophages and their ensuing differentiation into foam cells. Whether CD36 is operative in transporting oxLDL into beta cells is not known. Granted that such a role can be documented, putative harmful effects on beta cell function by intracellular accumulation of oxLDL could then be examined.

CD36 mediates the uptake of fatty acids into cells including beta cells (Su & Abumrad 2009). Previously, we investigated the impact of CD36 over-expression in the uptake of fatty acids and its relation to clonal beta cell function and metabolism (Wallin *et al.* 2010). Here we first tested for an effect of CD36 on the uptake of fluorescein isothiocyanate (FITC)-labeled oxLDL in clonal beta cells. To investigate the specificity of our model, we tested for effects of nLDL in parallel, the rationale being that nLDL, although with a broadly similar molecular structure, should not be scavenged by CD36. Upon documenting increased intracellular accumulation of oxLDL we explored the impact of such accumulation on clonal beta cell function and viability and also the relationship between oxLDL-associated ^{3}H-cholesterol cellular uptake and efflux. Furthermore, we tested for viability in experiments in which oxLDL uptake was reduced by the presence of a CD36-blocking agent. Lastly, we extended our findings to native beta cells by measuring relevant parameters in pancreatic islets of wild-type CD36 and CD36$^{-/-}$ mice.

Materials and methods

Cell lines and culture

Rat insulinoma INS-1 cell-derived clones were cultured in RPMI 1640 containing 11 mmol/L glucose (Asfari *et al.* 1992) unless otherwise indicated. Establishment of a stable INSr9 clone carrying the reverse tetracycline/doxycycline-dependent transactivator (Gossen *et al.* 1995) as well as the second stable transfection with rat CD36, clone selection and screening was described previously (Wallin *et al.* 2010).

Mouse islets and culture

Male CD36$^{-/-}$ mice were kindly provided by Mikael Karlsson (Karolinska Institutet) originally created by Maria Febbraio (Febbraio *et al.* 1999), and parallel matched C57BL/6J mice were obtained from Charles River Laboratories (Sweden). Animal care and treatment followed the recommendations of Guide by the Stockholm North Committee for Experimental Animal Ethics (permit N145/14) and the Swedish National Board for Laboratory Animals. The committee specifically approved the study. Islets of Langerhans were isolated from CD36$^{-/-}$ and control mice (3–4 months old) by collagenase digestion in Hanks' balanced salt solution, followed by sedimentation. Islets were then cultured for 48 h at 11 mM glucose in RPMI 1640, 1% FCS ± 100 µg/mL oxLDL or nLDL. After culture, islets were used for different experiments (see below).

CD36$^{-/-}$ mice: general characteristics

CD36$^{-/-}$ mice displayed lower weight and lower P-glucose, confirming a previous report (Hajri *et al.* 2002) (Supplementary data, see section on supplementary data given at the end of this article).

Preparation of lipoproteins

Lipoproteins were isolated as described (Havel *et al.* 1955). For further details, see Supplementary data.

Preparation of oxLDL

Oxidized LDL was obtained by incubating 1 mL of LDL (1 mg/mL protein content, determined by Bradford assay, Bio-Rad) with 20 µmol/L $CuSO_4$ for 18 h at 37°C. The oxidation of LDL leads to the formation of lipid peroxidation products, that is MDA, that are reactive to thiobarbituric acid (TBARS). The extent of oxidation was evaluated as described (Puhl *et al.* 1994). The oxLDL preparations showed high concentrations of TBARS (>60 nmol/mg protein). Very low or no detectable levels of TBARS were found in nLDL preparations (Supplementary Fig. 1).

Preparation of FITC-labeled LDL

LDL(oxLDL and LDL) was labeled by a modification of a previously described method (Schmitz *et al.* 1987). For details, see Supplementary data.

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Uptake of FITC-labeled LDL

Cells were seeded in 24-well plates. After 24 h of culture, the media were changed and cells were cultured for 36 h in 10% FBS ± doxycycline (Dox, 75 or 500 ng/mL). They were then further cultured in media containing only 1% FBS for 12 h ± Dox (75 or 500 ng/mL). Fresh culture media with 1% FBS ± Dox (75 or 500 ng/mL) ± LDLs (20 µg/mL) were added. In some experiments also SSO in the concentration of 200 µmol/L was added. After 30 min and onward time-points, cells were washed twice with ice cold PBS. Cells were then flushed off by vigorous pipetting, transferred to tubes and centrifuged at 123 ***g*** for 2 min at 4°C. Supernatants were discarded and cells re-suspended in 4% formaldehyde. The uptake of FITC-labeled nLDL and oxLDL was analyzed on a CyAnTM ADP flow cytometer (Dako). For each experiment 10,000 events were acquired. The fluorescence of cells without added lipoproteins or FITC was used as control. In some experiments the uptake of LDLs was also performed with INSr9 cells (which only harbor the doxycycline gene).

Confocal microscopy

After uptake of FITC-labeled LDLs for 24 h cells were transferred to slides by using Shandon Cytospin 2 at 500 rpm for 5 min. Nuclei were stained by DAPI (1:250,000) (Sigma-Aldrich) in PBS for 10 min. Slides were then washed twice in PBS and mounted. Cell fluorescence was analyzed with a Leica TCS SP5 (Leica Microsystems) microscope equipped with filters for the detection of DAPI and FITC. Images were acquired at ×63 magnification using an immersion oil objective and the Leica Application Suite Advanced Fluorescence software and mounted in Adobe Photoshop.

Viability assays

Cells were cultured in 96-well plates for 48 h ± Dox (500 ng/mL) and then further incubated for 48 h ± Dox (500 ng/mL) and with 20, 50 or 100 µg/mL nLDL or oxLDL. In some experiments 200 µmol/L SSO was present during the final 48 h of culture. For details about the MTT assay (Mosmann 1983) refer to Supplementary data. Apoptosis was quantified with a cell death detection ELISA^{plus} kit (Roche Applied Bioscience) according to manufacturer's instruction.

Insulin secretion and cellular content

Cells

Cells were seeded in 24-well plates. After 24 h, culture media were changed and cells were cultured for 48 h ± Dox (500 ng/mL). Cells were exposed to LDLs (20 or 50 µg/mL) either acutely (LDLs present only during final incubations) or longer term (LDLs present for 48 h prior to final incubations). Before final incubations cells were pre-cultured for 3 h in culture media without glucose together with 1% FBS, and additionally for 30 min in KRB buffer without glucose. Final incubations (30 min) were performed in KRB with 2.5 and 21.5 mmol/L glucose at 37°C. Aliquots of media were saved for insulin secretion. Cellular insulin content was extracted by acid ethanol as described (Wallin *et al.* 2010).

Islets

Islets (equal-sized) were pre-incubated as reported (Björklund & Grill 1993), followed by 60-min batch incubation (3 islets per tube in triplicate, 2.8 or 16.7 mmol/L glucose). Islets from the batch incubations were treated with acid-ethanol to extract cellular insulin (Björklund & Grill 1993). Insulin was measured by RIA as described (Herbert *et al.* 1965, Grill *et al.* 1990).

Radiolabelling of LDL: [1,2-^{3}H] cholesterol

LDL d(oxLDL and nLDL) was radiolabeled with [1,2-^{3}H] cholesteryl oleate as described (Faust *et al.* 1977). For details, see Supplementary data.

Uptake and efflux of ^{3}H-cholesterol LDLs

Cells were induced ± Dox (500 ng/mL) as for FITC experiments. The uptake and efflux of ^{3}H-labeled LDLs was assessed after culture with ^{3}H-cholesterol-nLDL or ^{3}H-cholesterol-oxLDL (20 µg/mL) for 24 h in 1% FBS. Media were saved and cells were then washed with PBS and lysed in acid ethanol. Aliquots of media and cell lysates were counted in a β scintillation counter. The protein content was measured by aliquots from each sample of lysates by the Bradford method. Results are presented as DPM/mg protein.

Mouse islets were cultured for 48 h in 1% FBS ± 100 µg/mL ^{3}H-labeled LDLs and uptake was measured as for cells (see above).

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Quantitative real-time PCR

Details are given in Supplementary data.

Statistical analysis

Results are expressed as mean ± S.E.M. Significant differences were tested by one-way ANOVA (for trend) and by two-way ANOVA and two-way ANOVA with repeated measures as indicated in the figure legends. Significances by ANOVA were followed by Tukey *post hoc* test. In experiments where results are expressed as per cent of control, 95% confidence intervals (CIs) were calculated. A P value <0.05 was considered significant.

Results

Over-expression of CD36 specifically enhances the uptake of FITC-labeled oxLDL in a dose-dependent manner

We investigated the uptake of FITC-labeled nLDL and oxLDLover a 48-h period using 500 and 75 ng/mL of Dox (Fig. 1A and B). Previously we reported that 75 ng/mL of Dox results in a 7.5-fold and 500 ng/mL in a 27-fold increase in CD36 protein (Wallin *et al.* 2010). The accumulation of oxLDL in non-induced INS-1 cells was only a fraction (less than half) of that found in CD36 over-expressing cells. CD36 over-expression induced by the lower concentration (75 ng/mL) of Dox led to less average fluorescent intensity than that obtained with exposure to the higher concentration (Fig. 1A and B). CD36 over-expression specifically increased uptake of oxLDL; the uptake of nLDL was thus not affected (Fig. 1C).

We performed experiments with INSr9 cells, which only harbor the tet-on gene. This was done in order to exclude an effect on uptake of FITC-oxLDL of Dox per se. Uptake of FITC-oxLDL in the INSr9 cells was not increased by exposure to Dox (Supplementary Fig. 2). Also, the ambient glucose concentration (tested at 2.5 and 21.5 mmol/L glucose during 6 h of FITC-LDLs exposure) did not affect the uptake of nLDL and oxLDL (Supplementary Fig. 3A and B, $n=1$).

Over-expression of CD36 increases intracellular accumulation of FITC-oxLDL

We used confocal microscopy to confirm the intracellular accumulation of FITC-oxLDL that was shown by flow cytometry (Supplementary Fig. 4). OxLDL-containing droplets (Supplementary Fig. 4 upper right and lower right panels) increased in CD36 over-expressing cells (Supplementary Fig. 4 lower right panel).

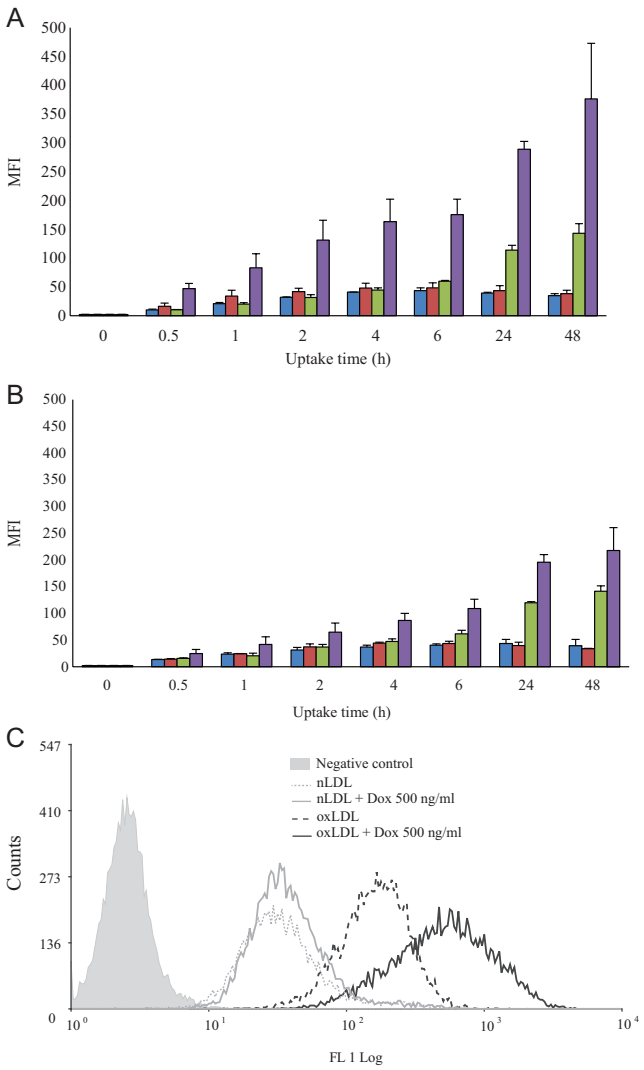

Figure 1
Induction of CD36 specifically and dose-dependently enhances cellular uptake of FITC-labeled oxLDL. Uptake of 20 µg/mL FITC-labeled nLDL or oxLDL ± 500 ng/mL Dox (A) or 75 ng/mL Dox (B), mean ± S.E.M. of five experiments (in duplicates) measured as mean fluorescence intensity (MFI) by FACS. In (A) and (B) $P < 0.05$ from 24 h for oxLDL + Dox vs all other conditions. Blue bars nLDL, red bars nLDL + Dox, green bars oxLDL, purple bars oxLDL + Dox. Flow cytometry data from the 48 h time point (with Dox 500 ng/mL) are depicted in (C), filled gray negative control, dotted gray line nLDL, gray line nLDL with Dox, dotted black line oxLDL, black line oxLDL with Dox.

Increased uptake of oxLDL is not linked to toxic effects

Having demonstrated that CD36 over-expression enhances oxLDL uptake by INS-1 cells, we could now examine whether such uptake exerts cytotoxic effects. Toxicity was

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evaluated by MTT, which provides a measure of oxidative metabolism (Janjic & Wollheim 1992) (Fig. 2A), and by the extent of apoptosis (Fig. 2B). Toxic effects by 50 and 100 but not at 20 µg/mL of oxLDL were detected (Fig. 2A and B). Importantly, CD36 over-expression and the consequent increased uptake of oxLDL failed to aggravate apparent toxicity (Fig. 2A and B).

Decreased uptake of oxLDL does not alleviate toxicity

We performed experiments with 100 µg/mL of oxLDL in the presence and absence of the CD36-blocking agent SSO (Supplementary Fig. 5). Expectedly, (Kuda *et al.* 2013) SSO inhibited FITC-labeled oxLDL uptake (by 50±8%, results not shown). However, inhibition of uptake did not alleviate toxicity: if anything exposure to SSO aggravated toxicity (viability being 48.9±5.6 vs 24.1±5.2% vs no oxLDL, Supplementary Fig. 5) (SSO per se did not negatively affect viability).

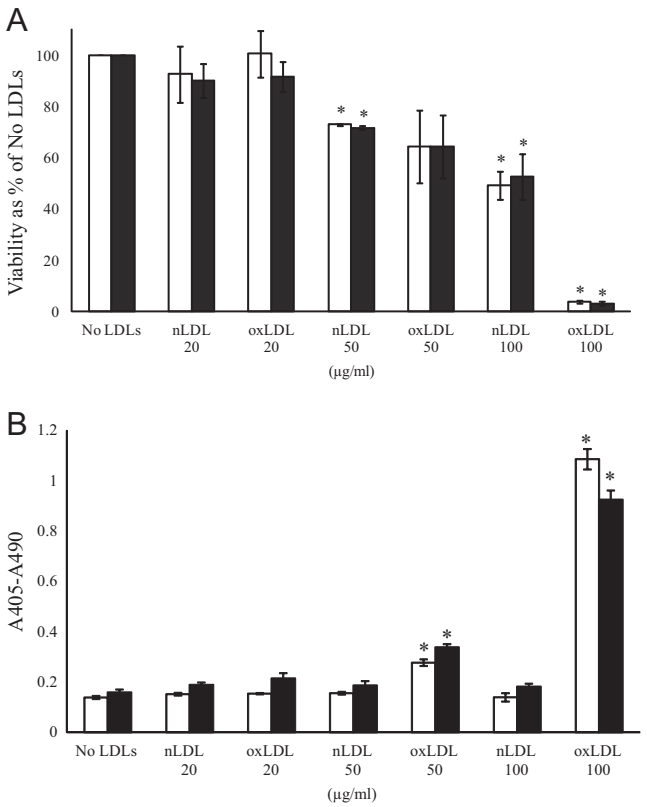

Figure 2
Increased uptake of oxLDL by CD36 over-expression does not exert toxic effects. Effects of nLDL and oxLDL on viability parameters were assessed by MTT (A) and cell death ELISA^{plus} apoptosis (B). Cells were cultured for 48 h with increasing concentrations of LDLs without Dox (white bars) and with 500 ng/mL Dox (black bars). Mean ± S.E.M. of three experiments (in duplicates), *$P < 0.05$, vs no addition of LDLs. In (A) 95% confidence intervals were calculated for treatment groups and compared to control, in (B) two-way ANOVA followed by Tukey *post hoc* testing was used.

Inhibitory effects of oxLDL on glucose-induced insulin secretion and cellular insulin content are not influenced by CD36 over-expression

We next evaluated the effects of increased oxLDL uptake on functional parameters, that is insulin secretion and content. Acute administration of oxLDL at 20 µg/mL, for 30 min inhibited glucose-induced insulin secretion (GSIS) to a minor extent in non-induced cells (Supplementary Fig. 6A and B).

Exposure for 48 h to 20 µg/mL oxLDL reduced glucose (21.5 mmol/L)-induced insulin secretion by 21±5% in the non-induced cells and by 33±9% in the CD36 over-expressing cells (Fig. 3A and C).

Exposure to the cytotoxic concentrations of 50 µg/mL of nLDL and oxLDL led to further inhibition of GSIS (Fig. 3B and D). Again, effects were found to be independent of CD36 over-expression.

Because a 48-h exposure to oxLDL had inhibitory effects on GSIS, we investigated if this effect was related to reduced cellular insulin content. At 20 µg/mL, oxLDL but not nLDL decreased cellular insulin content in both non-induced and CD36 over-expressing cells compared to cells

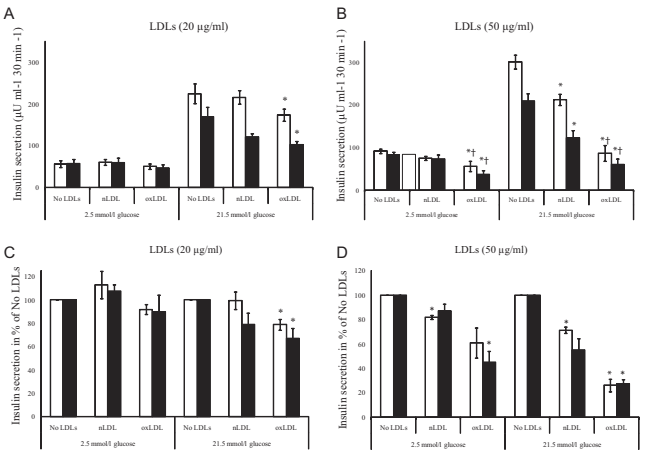

Figure 3
Effects of oxLDL on GSIS are not influenced by CD36 over-expression. Cells were cultured for 48 h in 20 µg/mL LDLs (A and C), $n = 7$ or in 50 µg/mL LDLs (B and D), $n = 3$ (in triplicates) followed by preincubation and final incubations for 30 min in 2.5 or 21.5 mmol/L glucose. White bars are cells cultured without, black bars with Dox (500 ng/mL). *$P < 0.05$ vs no LDLs. †$P < 0.05$ vs nLDL. (A and B) Show insulin secretion expressed as µU/mL/30 min; (C and D) show insulin secretion expressed in % of no LDLs. For the respective glucose concentrations two-way ANOVA followed by Tukey *post hoc* test was used in (A and B), whereas 95% confidence intervals were calculated for treatment groups and compared to control in (C and D).

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not treated with nLDL and oxLDL (Fig. 4A and C). The reduction in insulin content was more marked at 50 µg/mL oxLDL. The reduction was not aggravated by CD36 over-expression (Fig. 4B and D).

Treatment with doxycycline and LDLs reduces protein content in INS-1 cells

INS-1 cells were cultured as for uptake/efflux experiments and treated with different concentrations of LDLs for 48 h after which protein was measured. Dox in a concentration of 500 ng/mL reduced protein content by a mean of 43%. Also nLDL and in particular oxLDL dose-dependently reduced protein content (Supplementary Fig. 7, $n=6$). The relationship between protein contents with and without dox-induction was not altered by increasing the concentration of ox LDL (Supplementary Fig. 7). These inhibitory effects prompted a need to express uptake and efflux data in relation to protein content.

INS-1 cells over-expressing CD36 display increased uptake and efflux of ^{3}H-cholesterol-labeled oxLDL

To corroborate our findings with FITC-labeled nLDl and oxLDL we performed experiments using ^{3}H-cholesterol-labeled nLDL and oxLDL. Also, we wished to test whether increased efflux of cholesterol could balance increased lipid uptake, thereby counteracting any negative effects otherwise seen (Luo *et al.* 2010). In the absence of negative effects linked to increased uptake of FITC-labeled oxLDL we therefore investigated cholesterol uptake and efflux in INS-1 cells over-expressing CD36 using ^{3}H-cholesterol-labeled nLDL and oxLDL. These experiments were done in the absence as well as in the presence of HDL, known to enhance the efflux of cholesterol. We observed intracellular accumulation of ^{3}H-cholesterol-labeled oxLDL. In the absence of HDL and when normalized by cellular protein, ^{3}H-cholesterol-labeled oxLDL content was almost doubled in over-expressing cells vs not over-expressing cells (4619 ± 526 vs 2477 ± 232 dpm/mg protein, $n=6$ experiments from 3 passages, $P<0.01$ for difference) measured after 24 h of efflux. At the same time efflux into the culture medium was increased in CD36 over-expressing cells approximately 1.6-fold (from 469 ± 40 to 735 ± 70 dpm/mg protein, $n=6$ experiments from 3 passages, $P<0.01$ for difference) (Fig. 5B). The presence of HDL increased ^{3}H-cholesterol efflux (Fig. 5B) and at the same time decreased cellular ^{3}H-cholesterol contents (Fig. 5A). Notably, the ratio of efflux to content was constant so far that it was not affected by the dox-induced increase in cellular uptake (Table 1). As to nLDL, whereas HDL increased efflux and reduced cellular contents of ^{3}H-cholesterol, there was no effect by dox on efflux or contents of ^{3}H-cholesterol (Fig. 5C and D).

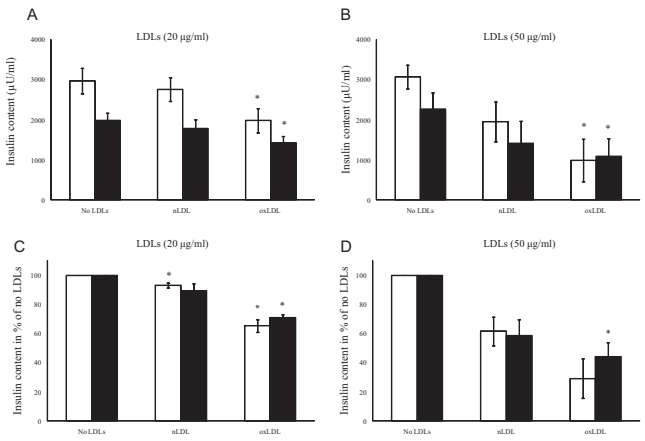

Figure 4
Effects of oxLDL on cellular insulin content are not influenced by CD36 over-expression. Results were obtained in the experiments described in Fig. 3 and similarly depicted.

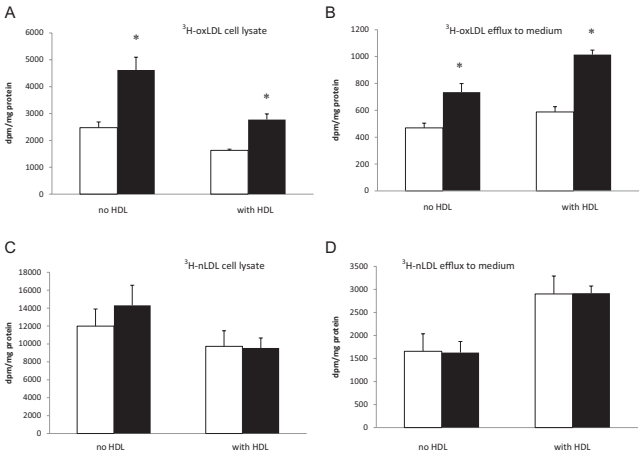

Figure 5
INS-1 cells over-expressing CD36 display increased uptake (A) and (C) and efflux (B) and (D) of ^{3}H-cholesterol-labeled oxLDL but not nLDL. Cells were cultured for 24 h with 20 µg/mL ^{3}H-cholesterol-LDLs without (white bars) and with (black bars) 500 ng/mL Dox and with and without 50 µg/mL HDL. (A) and (C) shows data from cell lysates (uptake) and (B) and (D) from culture medium (efflux), $n = 6$, $^*P < 0.05$, *t*-test.

nLDL and oxLDL both affect the expression of CD36 in INS-1 cells

Culture with 25 µg/mL nLDL and oxLDL for 2, 6, 24 and 48 h upregulated mRNA for CD36 (Supplementary Fig. 8). Maximum CD36 mRNA levels were seen after 6 and 24 h.

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Table 1 Efflux of cholesterol in % of cellular content is independent of Dox-induced increase in cellular uptake.

	Cholesterol in % of cellular content
Oxidized	
^{3}H-oxLDL	19 ± 0
^{3}H-oxLDL + DOX	16 ± 1
^{3}H-oxLDL + HDL	36 ± 2
^{3}H-oxLDL + HDL + DOX	36 ± 1
Native	
^{3}H-nLDL	17 ± 6
^{3}H-nLDL + DOX	12 ± 2
^{3}H-nLDL + HDL	31 ± 2
^{3}H-nLDL + HDL + DOX	32 ± 2

OxLDL increases CD36 expression in CD36 wild-type mice, whereas SRB is upregulated also in CD36$^{-/-}$ mice

Expression of CD36 in CD36 wild-type islets increased 2.4 times following culture for 48 h in 100 µg/mL oxLDL (Fig. 6). Expectedly, RT-PCR did not detect any expression of CD36 in CD36$^{-/-}$ islets. Another scavenger receptor, SRB1, which binds oxLDL (Moore & Freeman 2006) was upregulated both in wild-type and CD36$^{-/-}$ mice (RT-PCR and protein). Expression of other receptors (ABCA1 and FFAR1) were not affected (Fig. 6).

Decreased uptake of ^{3}H-cholesterol-labeled oxLDL but not nLDL in islets from CD36$^{-/-}$ mice

After culture in ^{3}H-cholesterol-labeled 100 µg/mL oxLDL or nLDL for 48 h, islets from CD36$^{-/-}$ mice displayed a modest decrease in the uptake of oxLDL vs islets from wt. animals. No decreased uptake was observed after culture with nLDL (Fig. 7).

In insulin secretion experiments (n = 4), we exposed isolated islets for 48 h to 20 µg/mL of oxLDL without any effects on insulin release (results no shown).

Discussion

We demonstrate the ability of the CD36 transporter/scavenger receptor to enhance the capacity of INS-1 insulin-secreting cells to take up oxLDL, an effect that correlated with the extent of over-expression of CD36. These CD36 effects were specific so that there was no effect on uptake of nLDL, a lipoprotein that is recognized as a target for scavenging receptors. The enhancing effect was robust so far that it seemed not to be dependent on the metabolic state of cells (thus not altered by low vs high glucose concentrations), nor was it associated with any effect by dox per se. Further, we confirm that oxLDL dose-dependently decreases viability as measured by MTT and apoptosis. Also insulin secretion and cellular insulin contents were reduced by oxLDL; these reductions could be functional but alternatively or additionally due to loss of cells, hence, secondary to an effect on viability. However, our main finding is that CD36 over-expression did not aggravate any of these negative effects, hence giving no indication for intracellular toxicity, at least in the time perspective tested here. Data from wild-type and CD36-knockout mice, though limited, seem at least compatible with the same conclusion also in pancreatic islets.

Thus, the negative effects of oxLDL do not associate with an increased uptake of FITC-labeled oxLDL in INS-1 cells. Likely, the toxic effects that we register (and then exclusively at high concentrations of oxLDL as also seen in previous studies, Okajima *et al.* 2005, Abderrahmani *et al.* 2007) are instead exerted extracellularly. Our results are in qualitative agreement with results in macrophages demonstrating that deleterious effects of oxLDL are not associated with internalization in these cells (Asmis *et al.* 2005). However, other reports, such as in retinal cells, indicate intracellular effects of CD36-induced increases of intracellular oxLDL (Gnanaguru *et al.* 2016). Hence, the question of extracellular vs intracellular effects of oxLDL has to be answered separately for different types of cells.

Extracellular effects could be mediated by several types of receptors for oxLDL; such receptors have been demonstrated in other tissues (Moore & Freeman 2006) and interactions have been linked to negative effects

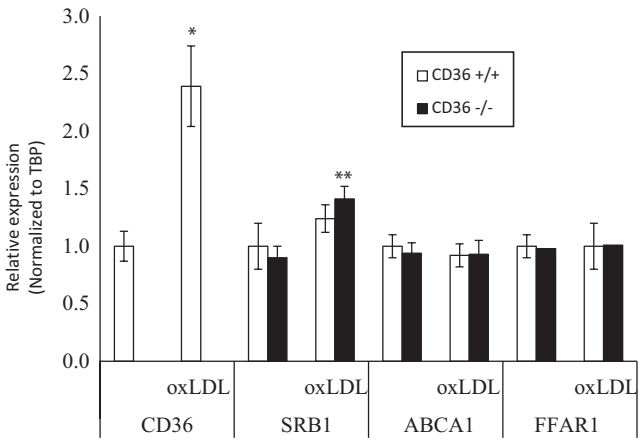

Figure 6
Quantitative RT-PCR showing gene expression of CD36, SR-B1, ABCA1 and FFAR1 (GPR40) mRNA in islets from CD36$^{-/-}$ and CD36^{-} wild type mice (n = 3 for CD36 and n = 5 for the other genes). Expression of CD36 in islets of CD36$^{-/-}$ was not detected. Expression was regularized to that of TATA Box Binding Protein (TBP). *P < 0.05 vs no LDL, **P < 0.01 vs no LDL, paired t-test.

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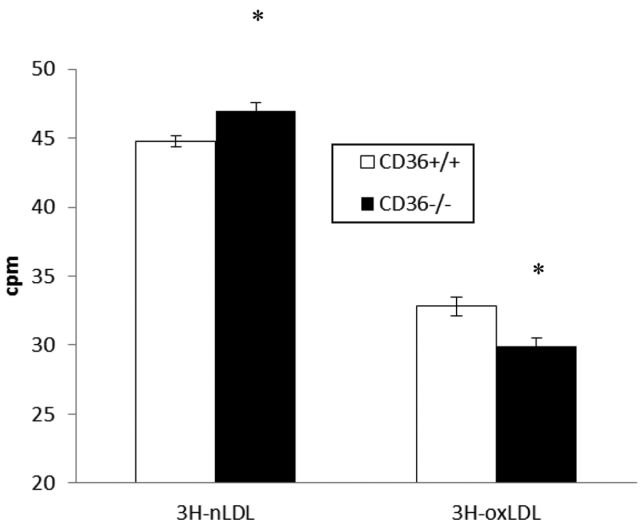

Figure 7
Uptake of ^{3}H-cholesterol-labeled oxLDL and nLDL in islets from CD36$^{-/-}$ and CD36 wild type mice after culture in 100 µmol/L for 48 h, *P = 0.15 vs wild type CD36 mice, One Way ANOVA, n = 4.

(Moore & Freeman 2006). Formation of cytotoxins acting on the cell surface could also be of importance (Asmis *et al.* 2005). Oxidative stress could have toxic effects and a rescue effect of an antioxidant, NAC, has been reported (Plaisance *et al.* 2016) a finding that could however not be reproduced during our experimental conditions (using NAC at a concentration of 1 mmol/L) (results not shown). A suggested impaired metabolism of lipids (Haefliger *et al.* 2013) should also be kept in mind. The exact mechanisms behind negative effects of high concentrations of oxLDL in beta cells remain to be investigated.

To corroborate our findings with FITC-labeled lipoproteins we measured in separate experiments the uptake and efflux of oxLDL ^{3}H-labeled cholesterol. In agreement with FITC data, also the uptake of oxLDL ^{3}H cholesterol of oxLDL was increased by CD36 over-expression. In addition, we find that the efflux of cholesterol appeared proportional to the degree of intracellular accumulation of cholesterol. Expectedly, the presence of HDL promoted efflux; efflux was however still proportional to the different amounts of intracellular ^{3}H-cholesterol as achieved by dox or no dox, and the uptake of oxLDL did not interfere with this process. One may also conclude that a pathological accumulation of cholesterol (Kruit *et al.* 2010) is, to some extent at least, counterbalanced by efflux, whether exerted by active mechanisms and/or by passive diffusion.

The FITC labels the protein part of LDL i.e. Apo lipoprotein B100 (ApoB100), whereas (^{3}H) is incorporated into the cholesterol part of LDL. Our finding that FITC-labeled products remain intracellularly over a prolonged period of time suggests that the protein part of LDL, ApoB100, is not rapidly metabolized by INS-1 cells. It has been shown that ApoB100-derived peptides can influence innate and adaptive immune responses (Nordin Fredrikson *et al.* 2003, Ketelhuth *et al.* 2011). Whether intracellular accumulation of ApoB100 can influence beta cell function in ways and at a time scale not tested here remains to be investigated.

We find, to our knowledge for the first time, that oxLDL upregulates mRNA expression of CD36 in islets from wild-type mice and that this finding can be reproduced in clonal beta cells (albeit with lesser specificity). Also in macrophages does oxLDL upregulate CD36 (Han *et al.* 1997). It seems possible this interaction between oxLDL and CD36 is a generalized one that can be extrapolated to other tissues as well. In further support of a role for CD36 in relation to the handling of oxLDL in native beta cells, we find that the uptake of ^{3}H cholesterol-labeled oxLDL in islets of CD36$^{-/-}$ vs wild-type islets was decreased. That the decrease was minor could possibly be due to upregulation of alternative transporter molecules, such as SRB1, as demonstrated here. Further, the extent of over-expression of CD36 vs no over-expression is likely to be more pronounced than the difference in wild type vs. knockout.

It is of interest that oxLDL toxicity was not reduced in islets from CD36$^{-/-}$ vs wild-type mice. Given a reduced uptake of oxLDL in islets from knockout mice, this finding is at least compatible with the general notion that oxLDL toxicity is mainly exerted extracellularly.

There are limitations of our study. We obtained the bulk of our data in clonal beta cells. These cells are highly differentiated; INS-1 cells displaying characteristics of insulin release that largely resemble those of native beta cells. However, the ongoing replication in clonal as opposed to native cells could possibly influence their response to lipoproteins. As to the preparation of oxLDL, it has been standardized and used in several studies during the last three decades. (And, according to the TBAR measurements should be classified as highly oxidized LDL.) However, we cannot rule out that other modification pathways, i.e. by HOCl, chloramines, phenoxyl radical intermediates and peroxynitrite (ONOO^{-}) (Berliner & Heinecke 1996), may generate forms of oxLDL which differ in terms of bioactivities vs the present preparation of oxLDL. Further, the possibility exists that the oxidative changes to LDL *in vivo* may differ from those that are induced here by copper. Lastly, there is insufficient knowledge about the correspondence between *in vivo* concentrations of oxLDL

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and those *in vitro* that here and elsewhere have been found to be beta cell toxic.

In conclusion, we demonstrate that CD36-dependent and specific uptake of oxLDL in clonal beta cells does not enhance oxLDL-induced toxicity. Also, our data highlight the presence of a working intracellular cholesterol efflux machinery that can balance increased uptake of the cholesterol moiety of oxLDL. Further, we present evidence for interactions of CD36 and oxLDL in pancreatic islet.

Supplementary data

This is linked to the online version of the paper at https://doi.org/10.1530/JME-18-0146.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

Establishment of INS-1 cells over-expressing of CD36 was performed in the lab of C B W by H W. A B designed the study and experiments were performed by Z M, T H and T W with help of D K and M F. Analysis of data was carried out by A B, T W and D K. A B wrote the manuscript and D K, V G and C B W edited the manuscript.

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