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Facilitation of fatty acid uptake by CD36 in insulin-producing cells reduces fatty-acid-induced insulin secretion and glucose regulation of fatty acid oxidation

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ABSTRACT

Facilitation of fatty acid uptake in beta cells could potentially affect beta cell metabolism and secretory function; however such effects have not been clearly documented. CD36 facilitates uptake of fatty acids (FA) in muscle and adipose tissue and is likely to exert a similar effect in beta cells. We investigated the impact of over-expressing CD36 on fatty acid uptake and beta cell function by a Tet-on system in INS-1 cells. Doxycycline dose-dependently increased the CD36 protein with localization mainly in the cell membrane. Over-expression increased both specific uptake and efflux of oleate whereas intracellular glycerides were only marginally increased and incorporation of ^{14}C-oleate or -palmitate into di- or triglycerides not affected. The normal potentiation of glucose-induced insulin secretion by acute addition of FA (50–100 μmol/l oleate and palmitate) was lost and the normal inhibitory effect of high glucose both on oleate oxidation and on the activity of carnitine palmitoyltransferase I was reduced. Over-expression did not induce apoptosis.
We conclude that induction of the CD36 transporter increases uptake of FA, the consequences of which are blunting of the functional interplay between glucose and FA on insulin secretion and oxidative metabolism.

1. Introduction

The importance of fatty acids (FA) for the function and survival of pancreatic beta cells is a controversial subject. A stimulatory effect by FA on insulin secretion was demonstrated many years ago [1,2]. It is also established that lipids within beta cells play a fuel-supply role during conditions of reduced availability of glucose [3]. The consequences of long-term elevated FA in vivo and in vitro are however not fully elucidated. For example, there is conflicting evidence whether long-term elevated FA give rise to "lipotoxicity", i.e. whether causing cell death by apoptosis [4,5] or only functional defects of varying severity [6,7]. Lack of consensus stems partly from the complex molecular interactions of FA with beta cells. Possible interactions range from proximal ones by un-metabolized FA interactions with G-protein-linked receptors [8] through diverse effects on metabolism and the production of signaling molecules [9], to distal interactions with the exocytosis of secretory granules [10].

However, most positive and negative effects by exogenous FA are envisaged to follow cellular uptake of FA. Extracellular elevated FA are expected to lead to increased uptake into cells because of passive concentration-dependent uptake. More recently it has been appreciated that uptake of FA is additionally regulated by active transport mechanisms that serve to enhance FA uptake. A FA transporter CD36 has been identified in several tissues, such as muscle, liver and platelets [11]. The importance of CD36 for FA metabolism and dyslipidemia has been demonstrated in the spontaneously hypertensive rat [12] and transgenic mice [13]. A role for CD36 for beta cell function was suggested by its demonstration in human pancreatic beta cells [14].

We hypothesized that over-expression of CD36 in beta cells would facilitate the uptake of FA also in beta cells and thereby lead to metabolic and functional alterations. To test this hypothesis we have used a Tet-on CD36 over-expressing cell line of INS-1 origin. INS-1 cells retain responsiveness to glucose in terms of insulin secretion and biosynthesis [15]; therefore the results obtained are expected to have relevance also to normal beta cells. To further increase relevance care was taken to use only concentrations of free i.e. not-protein-bound FA that are within the range of circulating concentrations seen in vivo.

2. Materials and methods

2.1. Establishment of INS-1 cells permitting inducible expression of CD36

Rat insulinoma INS-1 cell-derived clones were cultured in RPMI 1640 containing 11 mM glucose [15] unless otherwise indicated. The first step which establishes a stable clone INS-rβ or INSr9 cell line, which carries the reverse tetracycline/doxycycline-dependent

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transactivator [16] was described previously [17,18]. The plasmid used in the secondary stable transfection was constructed by subcloning the cDNA encoding the rat CD36 into the expression vector PUHD10-3 [16]. The procedures for stable transfection, clone selection and screening were previously described [17].

2.2. Cell culture

The CD36*10 clone was selected for further experiments. Cells were grown in monolayer cultures in RPMI 1640 with supplements as described [15] until 60–80% confluence. Cells were then exposed to doxycycline and/or FA at different concentrations in the presence of 10% FCS unless stated differently. Albumin-bound oleate was prepared by stirring the FA sodium salt at 45 °C with defatted BSA (Sigma). Sodium-palmitate was heated in NaOH (0.1 mol/l) to 100 °C until dissolved, and then drop wise added to stirring defatted BSA solution. The pH of the solutions was adjusted to 7.4 and was filtered.

2.3. Measurements of insulin secretion and cellular insulin content

Cells were seeded in 24-well plates and cultured for 48 h with and without doxycycline (500 ng/ml). Palmitate or oleate was added as indicated. Briefly, in *acute* experiments palmitate and oleate were added during 30 min final incubations whereas in *long-term* experiments palmitate or oleate were present during a 48 h culture period (together with doxycycline) prior to pre-incubation and final incubations. After culture cells were incubated for 2–3 h in RPMI with 1% FCS and 20 mM Hepes in the absence of glucose. Cells were then pre-incubated in KRB in the absence of glucose for 30 min, followed by final incubations in KRB medium, 10 mmol/l HEPES, 0.2% BSA at indicated concentrations of glucose and in acute experiments FA. Aliquots of media were collected for insulin assay. Insulin was extracted from the remaining cells by acid ethanol (1.5 ml HCl in 98.5 ml 70% ethanol) being added to each well. After storage at −20 °C overnight cells were scraped off and aliquots retrieved for measurement of insulin content.

Insulin was analyzed by RIA by using ^{125}I-labeled insulin and an antibody against porcine insulin established at the Dept. of Endocrinology, Karolinska Hospital [19,20].

2.4. Immunofluorescence and Western blotting

For immunofluorescence, cells grown on polyornithine-treated glass cover slips were treated for 24 h with or without doxycycline (500 ng/ml). Cells were then washed, fixed for 10 min in 4% paraformaldehyde (PAF) and permeabilized for 1 h in PBS containing 0.1% saponin and 0.5% BSA. The cells were incubated overnight at 4 °C with CD36 antibody (Cascade Bioscience USA, mouse monoclonal IgA, clone 63) at a dilution of 1:500. They were then exposed to the insulin antibody for 1 h at room temperature after which FITC- or Rhodamine-conjugated secondary antibodies were applied for 1 h at room temperature. Cover slips were mounted on glass slides with Vectastain (Reactolab, Lausanne, Switzerland). Samples were analyzed using a Zeiss laser confocal microscope (LSM 510, Zurich, Switzerland). Images were taken with a 60× objective.

For Western blotting cells were washed twice with ice-cold PBS. They were then processed basically as previously described [21]. A CD36 rabbit polyclonal antibody against amino acids 1-300 of human CD36 at a dilution of 1:500 (Santa Cruz Biotechnology, Santa Cruz, CA) was used as primary antibody. As second antibody an anti-rabbit IgG at the dilution of 1:5,000 was used.

2.5. Subcellular fractionation

Cells were grown with and without doxycycline as described above. After washing, cells were harvested and homogenized with a pestle motor homogenizator followed by 10 passages through a syringe in homogenization buffer followed by centrifugations as outlined in [22].

2.6. Fatty acid uptake and efflux

Cells were cultured in 75 cm^2 flasks for 48 h with and without doxycycline (500 ng/ml) until about 80% confluence. For uptake experiments, cells were trypsinized and washed in ice-cold KRB in the absence of glucose. Cells were counted in a Bürker chamber, and an equal amount of cells was transferred to new tubes and centrifuged. Cells were re-suspended in ice-cold KRB with 11 mmol/l glucose and 2.1 μCi (6 μmol/l) ^{14}C-oleate (GE Healthcare) and transferred in triplicates to microcentrifugation tubes, which were prepared with a bottom layer of 6 mol/l urea solution over layered by a 10:3 mixture of dibutyl-:dinonylphtalate [23]. Uptake was terminated by centrifugation at 8000 rpm for 15 s. The urea layer containing the cells was cut off and put into scintillation vials. Scintillation liquid was added and samples were transferred to a gamma counter (1900TR Liquid scintillation analyzer, Packard).

In some uptake experiments an inhibitor of CD36 Sulfo-*N*-Succinimidyl-Oleate (SSO) was used. SSO (a kind gift from Dr. N. A. Abumrad, Washington University School of Medicine) was prepared in 1 M stock solution in DMSO. Cells were pre-incubated for 25 min in 37 °C with KRB without glucose and with and without 0.5 mmol/l SSO. The DMSO concentration was kept below 0.05% and was added at the same concentration to control experiments. Cells were washed 3 times with KRB without glucose, trypsinized and further treated as above.

For efflux experiments cells were trypsinized and washed in KRB in the absence of glucose. Cells were then incubated with KRB and ^{14}C-oleate (same concentration as for uptake experiments) for 4 min in room temperature. Cells were then centrifuged and re-suspended in ice-cold 2% BSA KRB and transferred to micro-centrifugation tubes. The efflux process was stopped at different time points by centrifugation at 8000 rpm for 15 s. The remaining radioactivity in the separated cells was processed as above. Aliquots from efflux media were subjected to thin layer chromatography (TLC) as detailed below (Section 2.10).

2.7. Staining of lipid accumulation by Oil Red O and measurements of triglyceride content

For Oil Red O staining cells were grown on polyornithine-treated glass cover slips. After 3 days of culture media were changed and cells were cultured for another 48 h in 0, 75, 150 or 500 ng/ml of doxycycline with and without palmitate (100 μmol/l). Cells were then fixed in 4% PAF, rinsed, exposed to a working solution made by mixing 8 ml H_2O with 12 ml stock solution (500 mg Oil Red O in 60% triethylphosphate) for 30 min and finally rinsed 10 times with water. Lipid droplets were visualized using confocal fluorescence microscopy (UltraView ERS, Perkin Elmer, USA).

Cellular triglycerides were extracted according to Folch [24] and measured using the Triglyceride (GPO-TRINDER) kit (Sigma).

2.8. Cell counting

Cells were seeded in 24 well plates. After 48 h of culture media were changed and cells were cultured for another 72 h in the presence of 50 μM palmitate or 100 μmol/l oleate with and without doxycycline (500 ng/ml). Cells were then trypsinized and counted in triplicates in a Bürker chamber.

2.9. DNA laddering

DNA fragmentation was assessed by the Quick Apoptosis DNA Ladder Detection Kit (LabForce/MBL, Nunningen, Switzerland) following the manufacturer's protocol.