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Primary hypercholesterolaemia impairs glucose homeostasis and insulin secretion in low-density lipoprotein receptor knockout mice independently of high-fat diet and obesity

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ABSTRACT

We investigated whether primary hypercholesterolaemia per se affects glucose homeostasis and insulin secretion in low-density lipoprotein receptor knockout mice ($LDLR^{-/-}$). Glucose plasma levels were increased and insulin decreased in $LDLR^{-/-}$ compared to the wild-type mice. $LDLR^{-/-}$ mice presented impaired glucose tolerance, but normal whole body insulin sensitivity. The dose–response curve of glucose-stimulated insulin secretion was shifted to the right in $LDLR^{-/-}$ islets. Significant reductions in insulin secretion in response to L-leucine or 2-ketoisocaproic acid were also observed in $LDLR^{-/-}$. Islet morphometric parameters, total insulin and DNA content were similar in both groups. Glucose uptake and oxidation were reduced in $LDLR^{-/-}$ islets. Removal of cholesterol from $LDLR^{-/-}$ islets corrected glucose-stimulated insulin secretion. These results indicate that enhanced membrane cholesterol content due to hypercholesterolaemia leads to a lower insulin secretion and glucose intolerance without affecting body insulin sensitivity. This represents an additional risk factor for diabetes and atherosclerosis in primary hypercholesterolaemia.

1. Introduction

The majority of plasma cholesterol is transported by low density lipoprotein (LDL) and high density lipoprotein (HDL) in mammals. It is well established that an elevated plasma concentration of LDL is a condition sufficient to trigger the development of atherosclerosis in humans and in animal models [1,2]. The low density lipoprotein receptor (LDLR) removes cholesterol-rich intermediate density lipoproteins (IDL) and LDL from the plasma compartment, thereby regulating plasma cholesterol levels [1]. Genetic defects in the LDLR cause familial hypercholesterolaemia (FH) in humans [3], rhesus monkeys [4], Watanabe heritable hyperlipidaemic rabbits [5] and genetically modified mice [2,6]. FH is one of the most frequent hereditary dominant disorders. Over 600 mutations of the human LDLR gene have been identified [7] and the prevalence of the heterozygous form of FH is estimated at ~1:500, although it may be higher in populations with founder effects [8].

Patients with type 2 diabetes mellitus present a high incidence of atherosclerosis complications [9] and a 2 to 4 fold increase in mortality rate, compared to the general population [10,11]. Diabetic patients exhibit dyslipidaemia characterized by hypertriglyceridaemia, increased hepatic secretion of very low density lipoprotein (VLDL), decreased HDL and increased and abnormal (glycated and/or oxidized) small dense LDL particles [12,13]. On the other hand, a deleterious effect of high fat diets on glucose homeostasis and diabetes predisposition has been demonstrated in several models [14–16]. When fed on a high-fat diet (western type) for 16–18 weeks, in addition to severe hyperlipidaemia and atherosclerosis, LDLR knockout mice ($LDLR^{-/--}$) develop obesity and diabetes, characterized by hyperglycaemia, hyperinsulinaemia and peripheral insulin resistance [17].

While diabetes induced dyslipidaemia [18] and high-fat diet induced disturbances in glucose homeostasis [15,19] have been extensively studied, the role of lipoproteins in the beta cell function is less well understood. Rat and human beta cells internalize LDL and VLDL through the high affinity LDLR [20]. The native or oxidized LDL uptake by pancreatic beta cells can be harmful to these cells [21]. Human purified VLDL and LDL induced a dose-dependent rate of

Abbreviations: ITT, insulin tolerance test; KIC, 2-ketoisocaproic acid; KRBB, Krebs-Ringer bicarbonate buffer; MBCD, methyl-beta-cyclodextrin; OGTT, oral glucose tolerance test; RIA, radioimmunoassay

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apoptosis in insulin-secreting beta TC3 cell line and C57BL/6 mice islets with an important participation of the LDLR and c-Jun N-terminal kinase signalling pathway [22]. In human pancreatic beta cells, LDL and VLDL uptake may contribute to intracellular lipid accumulation, a process more evident in the aging beta cell population [23]. Previous *in vitro* studies in other cell types have shown that oxidized LDL exerts cytotoxic effects through the formation of oxidized lipids [24]. In this regard, LDLR$^{-/-}$ mice constitute a useful model for studying the association between primary hypercholesterolaemia and putative alterations of insulin secretion.

In this study, we investigated whether primary hypercholesterolaemia *per se*, without other metabolic confounding factors generally associated with high fat diets or metabolic syndrome, affects glucose homeostasis and insulin secretion in low fat fed LDL receptor knockout mice.

2. Materials and methods

2.1. Animals

Animal experiments were approved by the university's Committee for Ethics in Animal Experimentation (CEEA/UNICAMP). C57Bl6 wild type (WT) control and LDL receptor deficient mice (LDLR$^{-/-}$, originally from Jackson Laboratory, Bar Harbor, ME) were obtained locally from colonies maintained at the University Multidisciplinary Center for Biological Research in Laboratory Animals (CEMIB/Unicamp). The LDLR$^{-/-}$ and WT mice had access to standard laboratory rodent chow diet (Nuvital CR1, Colombo, Paraná, Brazil) and water ad libitum and were housed at 22 ± 1 °C on a 12 h light/dark cycle. All experiments were performed in both male and female wild type and knockout mice. Since no significant differences were observed between sexes, data presented here are from pooled results. Results separated by sex are provided as supplementary material.

2.2. Plasma biochemical analysis

Blood samples were collected from either the retro-orbital plexus or the tail tip of anesthetized mice. Total cholesterol (CHOL), triglycerides (TG) and non-esterified fatty acids (NEFA) were measured in fresh plasma in the fasting state (12 h) using standard commercial kits, according to the manufacturer's instructions (Boehringer Mannhein®, Germany; Merck®, Germany and Wako®, Germany, respectively). Glucose levels were measured using a glucose analyzer (Accu-Chek Advantage, Roche Diagnostic®, Switzerland) and plasma insulin was measured by radioimmunoassay (RIA) using rat insulin standard. Both male and female mice of 16–20 weeks of age were used for the experiments. Animals were fasted for overnight or had free access to food (fed) before blood sampling, which was carried out between 8:00 and 9:00 am.

2.3. Pancreas morphometry and immunohistochemistry

For morphometric analysis, pancreases from control and knockout animals were removed, weighed and fixed overnight with Bouin fixative solution. After fixation, each pancreas was cut in five pieces of similar sizes. All the pancreas fragments were embedded in paraffin and three fragments were selected in a systematic manner [25] (fragments 1, 3 and 5). From each fragment block, exhaustive 5 μm serial sections were obtained (every 20th sections) and two of these sections were randomly selected and then processed for the insulin immunoperoxidase reaction, as previously described [26]. All islets from the six sections processed for each animal were covered systematically by capturing images with a digital camera (Nikon FDX-35) coupled to a Nikon Eclipse E800 microscope. The islet, B-cell and section areas were analyzed using the free software Image Tool (http://ddsdx.uthscsa.edu/dig/itdesc.html). As previously described [27], the percentage of beta cell area in the islet was calculated by dividing the area of all insulin-positive cells by the islet area and by multiplying by 100. The islet mass was calculated by multiplying the pancreas weight by the percentage of the total islet area per pancreas section. The beta cell mass was calculated by multiplying the islet mass by the percentage of beta cells per islet. Immunofluorescence for insulin and glucagon was also performed in cryostat sections of pancreas from control and knockout animals, as previously described [26]. Labelling was observed and documented using a confocal laser scanning microscope (Zeiss LSM 510 META, Germany).

2.4. Oral glucose tolerance test (OGTT) and insulin tolerance test (ITT)

Mice were submitted to an OGTT at 16 weeks of age. Mice were fasted for 12 h and a basal blood sample was harvested from the tail tip ($t = 0$ min). A glucose load of 1.5 g/kg body weight was then administered by oral gavage and additional blood samples were collected at 15, 30, 60 and 90 min [17]. For the ITT, fed mice were injected with 0.5 U/kg body weight of human insulin (Biohulin®R, Biobrás, Brazil), i.p. Blood was taken immediately before insulin injection and at the times 10, 15, 30 and 60 min for glucose analysis. Results were expressed as percentage of initial blood glucose concentration. Glucose levels during OGTT and ITT were determined immediately, as described above.

2.5. Pancreatic islet isolation, static insulin secretion, and islet insulin content

The pancreatic islets were isolated from fed mice (20 week-old) by collagenase digestion and then selected with a microscope to exclude any contaminating tissues [28]. After isolation, batches of four islets each were pre-incubated in Krebs-Ringer bicarbonate buffer (KRBB) containing, in mM: 115 NaCl, 5 KCl, 24 $NaHCO_3$, 2.56 $CaCl_2$, 1 $MgCl_2$ and 25 Hepes, pH 7.4 plus 2.8 mM glucose and 0.3% Bovine Serum Albumin (BSA) for 30 min at 37 °C. The islets were further incubated for 1 h in the presence of increasing concentrations of glucose (2.8–27 mM) or 2.8 mM glucose plus L-leucine (10 mM) or 2-ketoisocaproic acid (KIC, 10 mM). In another set of experiments, islets were pre-incubated in the presence of 10 mM methyl-beta-cyclodextrine [29] for 1 h and then exposed to 2.8 or 11.1 for an equal period. Total islet insulin content was extracted using the acid/ethanol solution followed by insulin RIA.

2.6. Perfusion studies

Groups of 50 islets from WT and LDLR$^{-/-}$ mice were placed on a Millipore SW 1300 filter (8 μm pore) in a perfusion chamber (four chambers for each perfusion) and perfused in a KRBB buffer for 100 min at a flow rate of 1 ml/min. Glucose (2.8 mM) was present from the onset of the experiment and was elevated to 11.1 mM glucose from the 60th min onward. Solutions were gassed with 95% O_2/5% CO_2 and maintained at 37 °C. Samples were collected from min 32 until the end of the perfusion period. Insulin released into the medium was measured by RIA.

2.7. Glucose uptake

Batches of 50 islets were incubated in KRBB containing 11.1 mM glucose in the presence of 2-deoxy-D-[U-^{14}C] glucose (2 μCi) for 1 h, after which the islets were washed twice with KRBB at 4 °C, precipitated and homogenized in the presence of 100 μl Trizol (Invitrogen), mixed with 6 ml of scintillation fluid to determine the radioactivity in a Beckman beta counter. Results are expressed as pmol/islet.h.

2.8. Glucose and L-leucine oxidation

Batches of 20 islets each were incubated in KRBB containing 11.1 mM glucose in the presence of D-[U^{14}-C] glucose (0.6 μCi) or 2.8 mM glucose plus 10 mM L-leucine in the presence of L-[U^{14}-C] leucine (0.35 μCi) to measure $^{14}CO_2$ production. Sealed rubber lined capped vials containing opened microtubes with the islets were incubated for 2 h in a shaking water bath at 37 °C. Two-hundred microliters of 1 N HCl was injected through the rubber caps into the microtubes containing the islets to stop respiration and 200 μl of 1 N NaOH was added to the vial to absorb CO_2. The vials were incubated without shaking at room temperature for an additional hour. Scintillation fluid was then added to the vials and radioactivity representing $^{14}CO_2$ production determined with a scintillation counter.

2.9. Islet cholesterol assay and methyl-β-cyclodextrin-cholesterol depletion

Cholesterol content was quantified by a fluorometric method using an enzyme-coupled reaction provided by the Amplex Red Cholesterol Assay kit (Molecular Probes) as previously described by Hao et al. [29]. Groups of 10 islets per mice were subjected to lipid extraction with chloroform/methanol (2:1; vol/vol), dried down under N_2 stream, and resuspended in 60-μl of working solution (Amplex Red Cholesterol Assay kit) supplemented with 0.1% Triton X-100. From that, 50 μl was used for cholesterol assay.

For cholesterol depletion, islets were incubated with 10 mM methyl-β-cyclodextrin (Sigma) at 37 °C for 1 h, which removed cholesterol from both plasma membrane and intracellular stores.

2.10. Statistical analysis

Results are presented as means ± SEM for the number of mice (n) indicated. When working with islets, n refers to the number of experiments performed with groups of four islets each. The data were analyzed by unpaired Student's t-test for two mean comparisons and two-way ANOVA (with Neuman–Keuls post-hoc test) for glucose tolerance experiments (repeated measures) and for dose–response experiments. The level of significance was set at $p<0.05$.

3. Results

3.1. Plasma lipids, glucose and insulin levels

Cholesterol (CHOL) and triacylglycerol (TG) levels were 2.7- and 1.8-fold higher in LDLR$^{-/-}$ mice than in WT mice ($p<0.05$), whereas non-esterified fatty acids (NEFA) level was not different between the two groups (Table 1). Fasting glucose level was slightly (5%), but significantly, higher in LDLR$^{-/-}$ than in wild-type (WT) mice and still within the normal range. This difference was probably only detected due to the high number of mice studied ($n=28$–31); however, in the fed state, plasma glucose levels were more markedly increased (20%) in the LDLR$^{-/-}$, compared to the WT mice ($p<0.05$). These findings in knockout mice may be explained by the significantly lower plasma insulin levels in both fasting (44%) and fed (36%) states when compared to the WT group ($p<0.05$) (Table 1).

Table 1
Plasma lipids, glucose and insulin levels in fasted and fed wild type (WT) and LDL receptor knockout (LDLR$^{-/-}$) mice.

		WT	n	LDLR$^{-/-}$	n
CHOL (mg/dl)		105 ± 2.6	(22)	280 ± 6.4*	(22)
TG (mg/dl)		104 ± 4.9	(22)	188 ± 7.0*	(22)
NEFA (mmol/l)		1.16 ± 0.04	(20)	1.12 ± 0.05	(19)
Glucose (mg/dl)	Fasted	71.7 ± 2.0	(31)	74.9 ± 1.5*	(28)
	Fed	121.0 ± 2.1	(41)	145.3 ± 2.4*	(38)
Insulin (pg/ml)	Fasted	278.7 ± 27.5	(18)	156.0 ± 9.4*	(20)
	Fed	688.5 ± 85.5	(16)	433.5 ± 38.4*	(14)

Data are means ± SEM of n (in parenthesis). *$p<0.05$ vs. WT. Animals were fasted for overnight or had free access to food (fed) before blood sampling which was done between 8:00 and 9:00 am. Body weight means for male WT and LDLR$^{-/-}$ are 29.0±0.8 and 24.0±0.2 g, respectively, and for female WT and LDLR$^{-/-}$ are 23.0±0.3 and 20.0±0.3 g, respectively ($p<0.05$ for both sex and genotype comparisons of body weights).

3.2. Glucose and insulin tolerance tests

In order to explain the higher glycaemia and lower insulinaemia in LDLR$^{-/-}$ mice, we performed OGTT and ITT in these mice. As shown in Fig. 1A, the increase in blood glucose concentration, induced after the oral glucose load, was similar between the two groups of mice at 15 min. However, from 30 until 90 min, blood glucose levels were significantly increased in LDLR$^{-/-}$ mice, compared with WT mice ($p<0.05$). The incremental area under the curve of the OGTT in LDLR$^{-/-}$ mice was significantly greater than that of WT mice (11,437 ± 441 vs. 6931 ± 482 mg/dl · 90 min for LDLR$^{-/-}$ and WT mice, respectively; $n=15$, $p<0.0001$). These results indicate that LDLR$^{-/-}$ mice are glucose intolerant. The i.p. administration of the insulin load for the ITT protocol resulted in similar plasma removal rates of glucose in both groups (Fig. 1B). These findings show that LDLR$^{-/-}$ mice are not insulin resistant and that whole body sensitivity to large doses of insulin is not different in LDLR$^{-/-}$ and WT mice. In order to confirm the lack of insulin resistance in these hypercholesterolaemic mice, we repeated the OGTT and ITT in aged (12 month old) mice. As shown in Fig. 1C and D, LDLR$^{-/-}$ mice maintain glucose intolerance (13,606 ± 1050 vs. 7292 ± 732 mg/dl · 90 min, $n=5$–7, $p=0.001$), but they still have normal insulin sensitivity at an advanced age (Fig. 1D).

3.3. Glucose-stimulated insulin secretion by isolated pancreatic islet

Since hyperglycaemic LDLR$^{-/-}$ mice were not insulin resistant, we next investigated the possibility that the pancreatic islet insulin secretory ability of LDLR$^{-/-}$ was reduced compared to islets from the WT mice. Batches containing four islets each from both groups of mice were incubated with increasing glucose concentrations (2.8–27.0 mM). Both groups of islets responded to increasing concentrations of glucose in a dose-dependent manner with an S-shaped pattern (Fig. 2). However, the dose–response curve was shifted to the right in islets from LDLR$^{-/-}$ mice, as compared to the islets from WT type. The half-maximal insulin-releasing glucose dose was 13.3 ± 0.6 and 10.9 ± 0.3 mM, for LDLR$^{-/-}$ and WT islets, respectively ($p<0.05$). At intermediate glucose concentrations (11.1 and 13.5 mM), the insulin secretion was significantly reduced in LDLR$^{-/-}$ islets when compared to the WT islets ($p<0.05$). These results suggest that the islets from LDLR$^{-/-}$ are less sensitive to glucose than islets from WT mice and could explain the lower insulinaemia found in these mice.

3.4. Pancreas morphometry, insulin and DNA islet content

Histological analysis of the pancreas revealed no significant differences between the control and knockout animals in terms of islet morphology and architecture: the islets were roughly spherical or oval in shape displaying a typical B-cell arrangement within the islet core and A-cells at the periphery (Fig. 3). Mass and area of pancreas, islets and beta cells were similar in LDLR$^{-/-}$ and WT mice (Table 2). Total islet insulin content (130.5 ± 13.1 and 105.6 ± 7.9 ng/islet for LDLR$^{-/-}$ ($n=10$) and WT ($n=8$) mice, respectively), as well as DNA content (2.33 ± 0.3 and 2.63 ± 0.4 ng/islet for LDLR$^{-/-}$ ($n=12$) and WT ($n=10$) mice, respectively), were also similar in both groups of mice.

3.5. Dynamics of insulin secretion

In order to confirm the previous experiment and analyze the insulin secretion in more detail, we determined the dynamics of

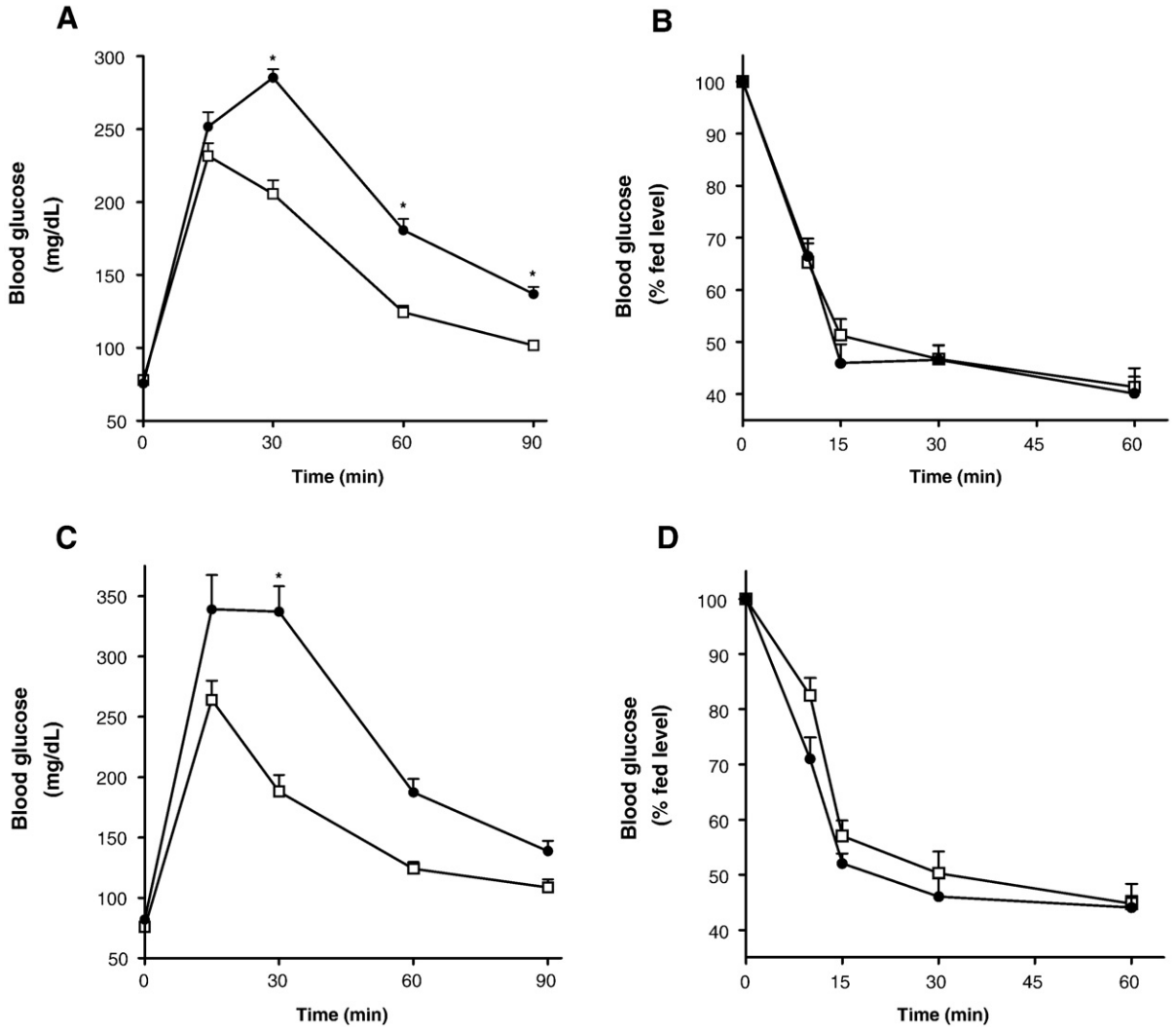

Fig. 1. Glucose tolerance test and insulin tolerance test in WT (*open squares*) and LDLR$^{-/-}$ (*solid circles*) in young (A and B) and aged (C and D) mice. Panels A and C: Fasted mice received an oral glucose load (1.5 g/kg) and blood samples were obtained from the tail tip at 0, 15, 30 and 60 min for glucose measurements. Panels B and D: Fed mice received an i.p. injection of regular insulin (0.50 U/kg). Blood samples were obtained from the tail tip at 0, 10, 15, 30 and 60 min for glucose measurements. Values represent the mean ± SEM of 6–15 mice per group. Two-way ANOVA (repeated measures): $p<0.0001$ for genotype, time and interaction; *$p<0.05$ or better vs. WT in each time point. Incremental area under the curve in A: 11,437 ± 441 vs. 6931 ± 482 mg/dl · 90 min for LDLR$^{-/-}$ and WT mice, respectively; $n=15$, $p<0.0001$; and in C: 13,606 ± 1050 vs. 7292 ± 732 mg/dl · 90 min, $n=5$–7, $p=0.001$.

glucose-induced insulin secretion by isolated islets along time using perfusion experiments (Fig. 4). In the presence of a non-stimulatory glucose concentration (2.8 mM), the insulin secretion was similar in both groups with a mean rate (32 to 60 min period) of 0.38 ± 0.13 and 0.34 ± 0.08 ng/50 islets · min^{-1} for LDLR$^{-/-}$ and WT groups, respectively. Challenge with 11.1 mM glucose induced a significant increase in insulin secretion rate in both types of islets. This secretion was characterized by a rapid first phase release (min 65–71), followed by a constant second phase (min 72–100) in both LDLR$^{-/-}$ and WT islets. The insulin release during the first phase was markedly lower in LDLR$^{-/-}$ than in WT islets. It reached a peak of 2.90 ± 0.26 ng/50 islets · min^{-1} in LDLR$^{-/-}$ islets, compared with 7.90 ± 0.50 ng/50 islets · min^{-1} in WT islets ($p<0.05$), representing increases of 8.5- and 20-fold compared to their respective basal values. During the second phase, the mean insulin secretion rate was also lower (60%) in LDLR$^{-/-}$ than in WT islets (0.52 ± 0.10 and 1.30 ± 0.30 ng/50 islets · min^{-1}, respectively; $p<0.05$).

3.6. L-Leucine and KIC stimulated insulin secretion

To determine whether the insulin secretory response is altered in the presence of other fuel secretagogues, the islets were incubated at 2.8 mM glucose in the absence and presence of 10 mM L-leucine or 10 mM KIC (Fig. 5). As in the previous experiments, the basal insulin release (2.8 mM glucose) was similar in both groups. However, when the LDLR$^{-/-}$ islets were exposed to the medium containing either L-leucine or KIC there were significant reductions in the LDLR$^{-/-}$ insulin response compared to WT islets. The amount of leucine and KIC stimulated insulin released by LDLR$^{-/-}$ represented 53% and 43% of that observed in WT islets, respectively ($p<0.05$).

3.7. Glucose uptake

The uptake of 2-deoxy-D-[U-^{14}C] glucose, dissolved in media containing 11.1 mM glucose, by LDLR$^{-/-}$ islets was approximately 20% lower than by WT islets during 1 h incubation. The values obtained were: 10.35 ± 1.07 and 12.84 ± 0.62 pmol/islet · h^{-1}, respectively for LDLR$^{-/-}$ and WT islets ($n=10$–14, $p<0.05$).

3.8. Glucose and L-leucine metabolism by pancreatic islet

Glucose and L-leucine oxidation, as judged by $^{14}CO_2$ production from D-[U^{14}-C] glucose and L-[U^{14}-C] leucine respectively, were measured in both LDLR$^{-/-}$ and WT islets. LDLR$^{-/-}$ islets showed

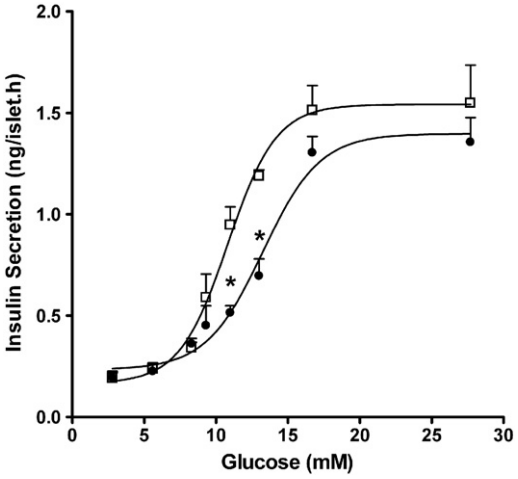

Fig. 2. Glucose-stimulated insulin secretion in islets from WT (*open squares*) and LDLR$^{-/-}$ (*solid circles*) mice. Each point represents mean ± SEM of 12 to 20 groups of four islets from five independent experiments. In each experiment we used two to three mice per group (WT and LDLR$^{-/-}$) to obtain enough islets, thus, a total of 10 to 15 mice per group were studied. Two-way ANOVA: $p<0.0001$ for genotype and glucose doses, $p<0.03$ interaction. *$P<0.005$ vs. WT for 11.1 and 13.5 mM glucose doses. The half-maximal insulin-releasing glucose dose was 13.3 ± 0.6 and 10.9 ± 0.3 mM, for LDLR$^{-/-}$ and WT islets, respectively ($p<0.05$).

a 30% reduction in glucose oxidation when compared to WT islets either after 1 or 2 h incubation periods: 8.5 ± 0.3 vs. 12.8 ± 1.3 pmol/islet · h^{-1}, respectively; $n=15$–16, $p<0.05$ and 20 ± 1.6 vs. 29 ± 1.8 pmol/islet · 2 h^{-1}, respectively; $n=14$–15, $p<0.05$. Glucose oxidation after 1 h incubation was also lower in LDLR$^{-/-}$ islets compared to WT islets. However, L-leucine oxidation was actually augmented (60%) in LDLR$^{-/-}$ islets compared to WT islets (4.2 ± 0.6 and 2.6 ± 0.3 pmol/islet · 2 h^{-1} for LDLR$^{-/-}$ and WT mice, respectively; $n=17$–18, $p<0.05$).

Table 2
Morphometric analysis of pancreas of wild type (WT) and LDL receptor knockout (LDLR$^{-/-}$) mice.

	WT	*n*	LDLR$^{-/-}$	*n*
Pancreas weight (mg)	168.4 ± 14	(5)	180.4 ± 6.4	(5)
Islet mass (mg)	0.95 ± 0.22	(5)	1.04 ± 0.13	(5)
Beta cell mass (mg)	0.66 ± 0.14	(5)	0.79 ± 0.10	(5)
Islet area (μm^2)	7026 ± 692.6	(251)	8136 ± 650.0	(264)
Beta cell area (μm^2)	5372 ± 504.6	(251)	6334 ± 534.1	(264)
Number of islet per section	8.37 ± 0.39	(5)	8.77 ± 0.46	(5)

Data are means ± SEM of *n* (mice or islet number). For parameters calculations see Materials and methods section.

3.9. Insulin secretion after cholesterol depletion

Although the genetic background from WT and LDLR$^{-/-}$ mice is the same (C57Bl6), since they are not littermates, some degree of DNA polymorphisms could differ between groups and raise some doubts on data interpretation. For this reason, we performed experiments designed to address the relevance of the hypercholesterolaemic phenotype. First we showed that the islet cholesterol content was 32% higher in LDLR$^{-/-}$ compared to WT islet (3.69 ± 0.42 vs. 2.80 ± 0.062 pg/10 islets, respectively, $n=5$, $p=0.031$). Next, we performed experiments depleting cholesterol from islets using methyl-beta-

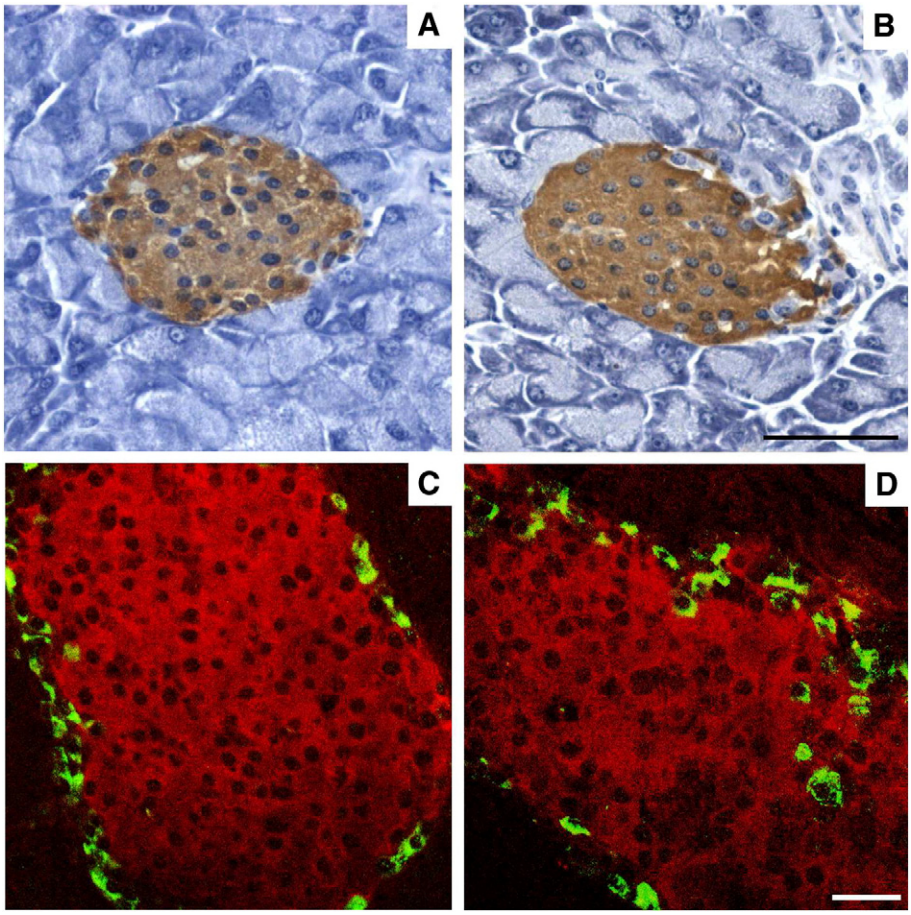

Fig. 3. Histological analysis of the endocrine pancreas from WT and LDLR$^{-/-}$ mice. WT (a,c) and LDLR$^{-/-}$ islets (b,d) were processed for insulin immunoperoxidase (a,b; scale 50 μm) or for double immunofluorescent detection of insulin (red) and glucagon (green) (c,d; scale 20 μm).

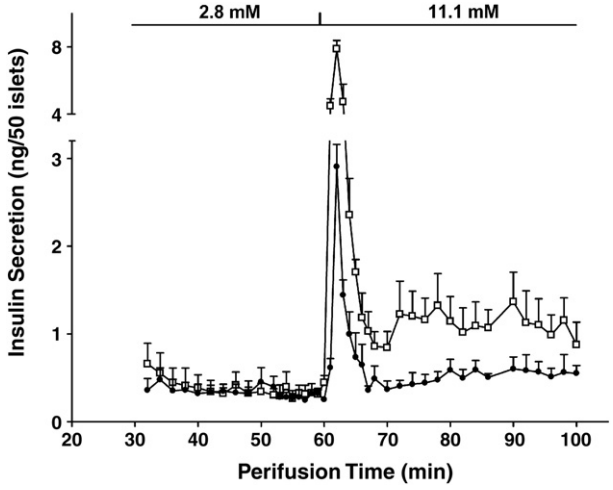

Fig. 4. Effects of 11.1 mM glucose on the dynamics of insulin release from WT (*open squares*) and LDLR$^{-/-}$ (*solid circles*) mice islet. Groups of 50 freshly isolated islets were perfused for 100 min. Glucose at 2.8 mM was present from the onset of the experiment and was increased to 11.1 mM from min 60 until the end of the perfusion period. Samples were taken from min 32 onward. Values are means ± SEM of four distinct experiments.

cyclodextrine (MBCD). Fig. 6 shows that cholesterol depletion improved insulin secretion in WT and LDLR$^{-/-}$ islets, either under low or intermediary glucose concentrations. At 2.8 mM glucose, significant increases of 75–90% were observed. At 11 mM glucose, the cholesterol depletion produced even more marked elevations in insulin secretion, 2.5 fold in WT and 4 fold increases in LDLR$^{-/-}$ islets, thus correcting completely the insulin secretion defect of LDLR$^{-/-}$ islet. Therefore, differences observed in insulin secretion were, in fact, due to increased islet cholesterol content rather than other putative genetic interferences.

4. Discussion

LDLR deficient mice are models of human FH in which LDLR are impaired or non-functional [3]. These mice show increased plasma cholesterol levels, almost exclusively in the IDL/LDL fraction [2]. In addition to hypercholesterolaemia, when submitted to a western type diet, LDLR$^{-/-}$ mice develop hypertriglyceridaemia and obesity [30], hyperglycaemia and insulin resistance [17], and severe atherosclerosis [31]. However, none of these disturbances, except the hypercholesterolaemia, have been described under a low fat balanced diet. In this study, we demonstrate, for the first time, that alterations in plasma glucose homeostasis and insulin secretion are induced by the hypercholesterolaemia in the absence of other metabolic factors induced by high fat or high carbohydrate unbalanced diets or obesity. Present data show that LDLR$^{-/-}$ mice fed a normal chow diet have hyperglycaemia and hypoinsulinaemia, glucose intolerance, and normal whole body insulin sensitivity. In addition, we also demonstrate that the main cause of the impaired glucose homeostasis is reduced pancreatic islet insulin secretion following fuel secretagogue stimuli. Since the maintenance of islet functionality is important for glucose homeostasis, these data indicate that hypercholesterolaemia per se can contribute to the onset of diabetes.

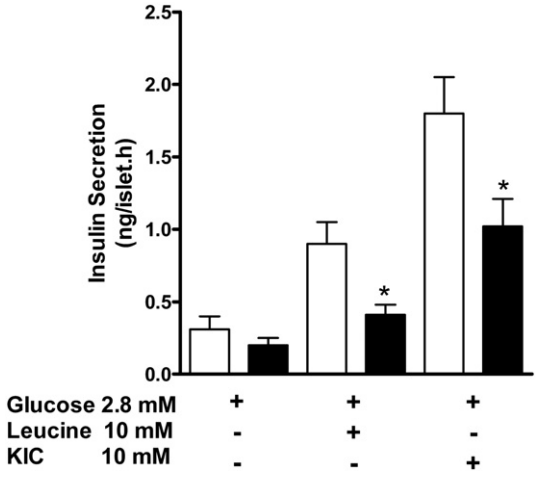

Fig. 5. Insulin secretion stimulated by L-leucine and 2-ketoisocaproic acid (KIC) in islets from WT (*open bars*) and LDLR$^{-/-}$ (*dark bars*) mice. Islets were incubated for 60 min with low glucose (2.8 mM) and low-glucose plus 10 mM L-leucine or KIC. The columns represent the means ± SEM (n = 15–20). *p<0.05 vs.WT.

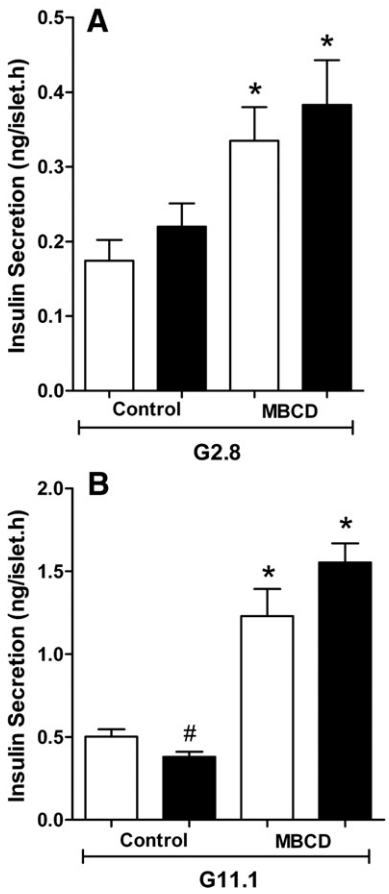

Fig. 6. Effects of cholesterol depletion on the insulin secretion by WT (*open bars*) and LDLR$^{-/-}$ (*dark bars*) islets under low (A, 2.8 mM) or intermediary (B, 11 mM) glucose concentrations. Cholesterol depletion was promoted by pre-incubating islets with 10 mM methyl-β-cyclodextrin (MBCD) for 1 h at 37°C. The columns represent the means ± SEM (n = 9–12). *p<0.05 MBCD vs. Control; #p<0.05, LDLR$^{-/-}$ vs. WT.

Human and rat beta cells express LDLR that can internalize lipoproteins, mostly LDL and VLDL [20]. In addition, rat beta cell necrosis can be induced by prolonged exposure to LDL in a manner dependent on LDL cellular binding and internalization [21]. Others have suggested that the exposure of beta TC3 cells and mouse pancreatic beta cells to VLDL and LDL increases beta cell apoptosis rate and reduces the level of the insulin gene transcription [22]. However, it is well known that LDL can become rapidly oxidized when exposed to several cell types in culture [32] and these cytotoxic and cell death effects may have been caused by oxidized instead of native LDL, particularly in islet cells that present low antioxidant capacity [33]. Indeed, in HIT-T15 cells, oxidized LDL reduced the expression of preproinsulin mRNA, the cellular insulin content, and the glucose stimulated insulin secretion [34]. Although the LDLR$^{-/-}$ mice cells do not take up native LDL, they may be susceptible to the harmful effects

of oxidized LDL in these mice [35], which are taken up by a distinct class of receptors, the scavenger receptors.

Our *in vivo* results show that islets from LDLR$^{-/-}$ mice have impaired insulin secretion in response to glucose without alterations in the pancreatic morphometric parameters, total insulin and DNA contents. These findings support the idea that the decreased response to glucose cannot be explained by differences in the islet size or number of beta cells, but that it is probably caused by a defect in the secretory process. We observed that LDLR$^{-/-}$ mice islets showed reduced first and second phases of insulin release compared with WT islets. The biphasic pattern of insulin secretion reflects the release of two different pools of granules. The first phase of insulin secretion is produced predominantly by ion fluxes and corresponds to the release of granules located near the plasma membrane, whereas the second phase results from the mobilization of granules located in a more internal reserve pool, whose release depends on fuel metabolism and Ca^{2+} concentrations but is also regulated by the cAMP and phosphoinositol pathways [36].

Although glucose is the main metabolic fuel that controls insulin synthesis and secretion, there are other metabolic substrates able to induce insulin release. Leucine is metabolized independently of glycolysis and stimulates insulin release by two intramitochondrial mechanisms: its own catabolism [37], and the allosteric activation of glutamate dehydrogenase that accelerate production of L-glutamate from L-glutamine. Both mechanisms lead to an increase in the metabolic flux rate through the Krebs cycle [38]. KIC has at least two metabolic fates in beta cells: it is completely oxidized, or it produces leucine from transamination of glutamate generating alpha-ketoglutarate [39]. The involvement of these two KIC metabolic pathways may explain why it is a more potent beta cell stimulator than leucine at equal molar concentrations (Fig. 5) [40,41].

The observation that islets from LDLR$^{-/-}$ mice released less insulin in response to L-leucine and KIC than WT islets, even with an increased metabolism of L-leucine in the islets from the knockout mice, is less easy to explain. Since intermediary substrates of Krebs cycle in LDLR$^{-/-}$ mice cells are reduced [42,43], the increased L-leucine metabolism in pancreatic islets could reflect a higher demand to replace this deficit. However, the increased leucine metabolism was not sufficient to normalize insulin secretion, suggesting additional downstream defects in LDLR$^{-/-}$ islet.

The reduced secretion in response to glucose in islets from LDLR$^{-/-}$ correlates well with the observed reduction in both glucose uptake and oxidation in these islets. Glucose stimulated insulin secretion is a complex process involving a cascade of regulatory factors and defective cholesterol homeostasis could result in partial loss of secretory function by several mechanisms. Recently, a connection between elevated serum cholesterol and reduced insulin secretion was reported for islets from severely hyperlipidaemic and obese mice (double mutant for apolipoprotein E and ob genes). Improved insulin secretion in these islets was observed after cholesterol depletion promoted by methyl-beta-cyclodextrin (MBCD) or by pharmacologic inhibition of cholesterol synthesis [29]. It was proposed that the excess of cell cholesterol inhibits insulin secretion by downregulation of glucose metabolism through a mechanism that impairs glucokinase activity [29]. Thus, a reduced glucose uptake, metabolism and insulin secretion observed in our experiments could also be a result of a higher amount of cholesterol in the LDLR$^{-/-}$ islets. Indeed, treatment of LDLR$^{-/-}$ islet with MBCD markedly increased and completely restored the insulin secretory capacity of LDLR$^{-/-}$ islet. Our data are also supported by others [44] who found that MBCD cholesterol depleted HIT-T15 cultured cells have increased insulin secretion associated with changes in the K^+ channel Kv2.1 function. On the other hand, more recent work showed that mouse beta cells treated with low dose MBCD presented a decreased membrane content of SNAP-25 (an exocytotic machinery component) and reduced insulin secretion [45]. In addition, inhibition of cholesterol synthesis with a squalene synthase inhibitor [46] in MIN6 cells and isolated islets resulted in impaired insulin secretion. This was associated with altered redistribution of several membrane ionic channels and secretory apparatus proteins. However, inhibition of cholesterol biosynthesis either with statins or squalene synthase inhibitors may have dose-dependent cytotoxic effects [47,48]. These studies and our results demonstrate that cholesterol is essential to maintain beta cell function, although the impact on the insulin secretion probably depends not only on how much of the membrane cholesterol content is altered but also on the particular cell compartment that is affected, either intracellular or surface cell membranes.

Altogether, our results strongly support the idea that the enhanced membrane cholesterol content resulting from primary moderate hypercholesterolaemia leads to a lower insulin secretion response to fuel stimuli. We showed, here in, that initial key steps involving glucose uptake and metabolism are impaired. Additional defects in more distal steps of the insulin secretion process in LDLR$^{-/-}$ mice islets, such as in membrane depolarization, transient Ca^{+2} fluxes or in the exocytotic machinery, may also be compromised. A great virtue of this *in vivo* model is the lack of confounding factors associated with unbalanced diets, high levels of NEFA or concurrent obesity which all lead primarily to insulin resistance and secondarily to pancreatic islet defects.

In conclusion, we demonstrate that moderate hypercholesterolaemia impairs the beta cell insulin secretion, leading to glucose intolerance without affecting peripheral insulin sensitivity. This represents an additional risk factor for developing diabetes and atherosclerosis.

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