METABOLISM CLINICAL AND EXPERIMENTAL 60 (2011) 1158–1164

available at www.sciencedirect.com

ScienceDirect

www.metabolismjournal.com

Lower expression of PKAα impairs insulin secretion in islets isolated from low-density lipoprotein receptor ($LDLR^{-/-}$) knockout mice

Maria Lúcia Bonfleur[a], *Rosane Aparecida Ribeiro*[b,*], *Sandra Lucinei Balbo*[a], *Emerielle Cristine Vanzela*[b], *Everardo Magalhães Carneiro*[b], *Helena Coutinho Franco de Oliveira*[b], *Antonio Carlos Boschero*[b]

[a] *Centro de Ciências Biológicas e da Saúde, Universidade Estadual do Oeste do Paraná (UNIOESTE), 85810-119 Cascavel, PR, Brazil*
[b] *Departamento de Anatomia, Biologia Celular e Fisiologia e Biofísica, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP), CEP 13083-970 Campinas, SP, Brazil*

ARTICLE INFO

Article history:
Received 20 August 2010
Accepted 16 December 2010

ABSTRACT

Hypercholesterolemic low-density lipoprotein receptor knockout mice ($LDLR^{-/-}$) show normal whole-body insulin sensitivity, but impaired glucose tolerance due to a reduced insulin secretion in response to glucose. Here, we investigate the possible mechanisms involved in such a defect in isolated $LDLR^{-/-}$ mice islets. Low-fat chow–fed female and male mice aged 20 weeks, $LDLR^{-/-}$ mice, and wild-type (WT) mice were used in this study. Static insulin secretion, cytoplasmatic Ca^{2+} analysis, and protein expression were measured in islets isolated from $LDLR^{-/-}$ and WT mice. At basal (2.8 mmol/L) and stimulatory (11.1 mmol/L) glucose concentrations, the insulin secretion rates induced by depolarizing agents such as KCl, L-arginine, and tolbutamide were significantly reduced in $LDLR^{-/-}$ when compared with control (WT) islets. In addition, KCl-induced Ca^{2+} influx at 2.8 mmol/L glucose was lower in $LDLR^{-/-}$ islets, suggesting a defect downstream of the substrate metabolism step of the insulin secretion pathway. Insulin secretion induced by the protein kinase A (PKA) activators forskolin and 3-isobutyl-1-methyl-xanthine, in the presence of 11.1 mmol/L glucose, was lower in $LDLR^{-/-}$ islets and was normalized in the presence of the protein kinase C pathway activators carbachol and phorbol 12-myristate 13-acetate. Western blotting analysis showed that phospholipase Cβ_2 expression was increased and PKAα was decreased in $LDLR^{-/-}$ compared with WT islets. Results indicate that the lower insulin secretion observed in islets from $LDLR^{-/-}$ mice at postprandial levels of glucose can be explained, at least in part, by the reduced expression of PKAα in these islets.

Institutional approval: All experiments were approved by the University's Committee for Ethics in Animal Experimentation (CEEA/UNICAMP).

Authors' contribution: Bonfleur ML and Ribeiro RA: conception and experimental design, execution of all experiments, analyses, data interpretation, and manuscript writing; Balbo SL and Carneiro EC: intellectual contribution along work development and manuscript writing; Vanzela EC: insulin secretion experiments; Oliveira HCF and Boschero AC: conception and experimental design, data interpretation, and manuscript writing.

* *Corresponding author*. Departamento de Anatomia, Biologia Celular e Fisiologia e Biofísica, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP), CP 6109, CEP 13083-970 Campinas, SP, Brazil. Tel.: +55 19 3521 6198; fax: +55 19 3521 6185.
E-mail address: rosaneribeirobio@yahoo.com.br (R.A. Ribeiro).

0026-0495/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.metabol.2010.12.010

1. Introduction

The incidence of type 2 diabetes mellitus (T2DM) has reached international epidemic dimensions and is expected to affect 300 million people within the next 25 years [1]. Numerous prospective cohort studies have indicated that T2DM is associated with a 3- to 4-fold increase in the risk for coronary heart disease (CHD) [2,3]. Patients with T2DM have 50% greater hospital mortality, and a 2-fold higher rate of death within 2 years post–myocardial infarction. Overall, CHD is the leading cause of death in individuals with T2DM [4].

Much of this increased CHD is associated with the presence of well-characterized risk factors, including abnormalities of plasma lipid and lipoprotein concentrations. Diabetic dyslipidemia is characterized by elevated blood levels of triglycerides (TG), low levels of high-density lipoprotein, and increased or normal concentrations of low-density lipoproteins (LDL) [5]. Chemically altered LDL (oxidized and glycated), as well as smaller and denser LDL particles, are also features of the diabetic dyslipidemia [6,7].

Modifications in lipoproteins observed in T2DM could contribute to the pathogenesis and progression of β-cell failure; however, the effect of dyslipidemias on β-cell function is poorly understood. The use of animal models that exhibit alterations in lipoprotein metabolism allows us to examine how factors that regulate lipid metabolism may influence susceptibility to T2DM. Hypercholesterolemic LDL receptor knockout mice ($LDLR^{-/-}$), a model of human familial hypercholesterolemia [8,9], when submitted to a Western-type diet, develop hypertriglyceridemia, obesity, hyperglycemia, insulin resistance [10], and severe atherosclerosis [11].

We previously demonstrated that $LDLR^{-/-}$, in the absence of a high-fat diet, shows normal whole-body insulin sensitivity, but impaired glucose tolerance due to a reduced insulin secretion in response to postprandial glucose concentrations [12]. In this study, we investigated the possible involvement of the protein kinase A (PKA) and phospholipase C (PLC)/protein kinase C (PKC) pathways in the reduced insulin secretion response to glucose found in hypercholesterolemic $LDLR^{-/-}$ mice islets.

2. Materials and methods

2.1. Materials

^{125}I human insulin was purchased from Amersham International (Little Chalfont, Bucks, UK). Routine reagents were purchased from Sigma Chemical (St Louis, MO).

2.2. Animals

The $LDLR^{-/-}$ mice founders were purchased from the Jackson Laboratory (Bar Harbor, ME). Control wild-type (WT) mice (C57BL/6 background) were obtained from the breeding colony of the State University of Campinas. Animal experiments were approved by the University's Committee for Ethics in Animal Experimentation. The mice had free access to standard laboratory rodent chow diet (Nuvital CR1, Colombo, PR, Brazil) and water ad libitum and were housed at 22°C ± 1°C on a 12-hour light/dark cycle.

2.3. General nutritional parameters

Fasted $LDL^{-/-}$ and WT mice were decapitated, their blood was collected, and plasma was stored at –20°C. Plasma glucose was measured using a glucose analyzer (Accu-Chek Advantage; Roche Diagnostic, Basel, Switzerland); insulin was measured by radioimmunoassay (RIA; as previously reported by Bonfleur et al [12]); and total cholesterol (CHOL), TG, and nonesterified fatty acids (NEFA) were measured using standard commercial kits, according to the manufacturer's instructions (Boehringer Mannhein, Germany; Merck, Germany; and Wako, Germany, respectively).

2.4. Pancreatic islet isolation and static insulin secretion

The pancreatic islets were isolated from fed mice (20 weeks old) by collagenase digestion and then selected with a microscope. After isolation, batches of 4 islets from each group were preincubated in Krebs-Ringer bicarbonate buffer (KRBB) containing the following (in millimoles per liter): 115 NaCl, 5 KCl, 24 $NaHCO_3$, 2.56 $CaCl_2$, 1 $MgCl_2$, and 25 HEPES (pH 7.4) plus 2.8 mmol/L glucose and 0.3% bovine serum albumin (BSA) for 30 minutes at 37°C. The islets were further incubated for 1 hour in KRBB containing different secretagogues, as indicated in the "Results" section. Aliquots of the supernatant at the end of the incubation period were kept at –20°C for posterior insulin measurement by RIA [13]. For islet insulin content, groups of 4 islets were collected and transferred to tubes of 1.5 mL. Deionized water (1 mL) was added to the samples, followed by disruption of the pancreatic cells using a Polytron PT 1200 C homogenizer (Brinkmann Instruments, NY); and the islet insulin content was measured by RIA.

2.5. Cytoplasmatic Ca^{2+} oscillations

Fresh pancreatic islets were incubated with Fura-2/AM (5 μmol/L) for 1 hour at 37°C in KRBB containing glucose (5.6 mmol/L), pH 7.4, and supplemented with BSA. Islets were washed with the same medium and placed in a chamber that was thermostatically regulated at 37°C on the stage of an inverted microscope (Nikon UK, Kingston, UK). Islets were then perifused with KRBB continuously gassed with 95% O_2/5% CO_2, pH 7.4, containing 2.8 mmol/L glucose with or without 40 mmol/L KCl. A ratio image was acquired at approximately every 5 seconds with an ORCA-100 CCD camera (Hammamatsu Photonics Iberica, Barcelona, Spain), in conjunction with a Lambda-10-CS dual-filter wheel (Sutter Instrument, Novato, CA), equipped with 340 and 380 nm, 10-nm bandpass filters, and a range of neutral density filters (Omega Opticals, Stanmore, UK). Data were obtained using the ImageMaster3 software (Photon Technology International, Pemberton, NJ).

2.6. Western blotting

Isolated islets from $LDLR^{-/-}$ and WT mice were homogenized in a buffer containing the following (in millimoles per liter): 100 Tris (pH 7.5), 10 sodium pyrophosphate, 100 sodium

fluoride, 10 EDTA, 10 sodium vanadate, 2 phenylmethylsulfonyl fluoride, and 1% Triton-X 100. The islets were disrupted using a Polytron PT 1200 C homogenizer (Brinkmann Instruments), using three 10-second pulses. The extracts were then centrifuged at 12 600*g* at 4°C for 5 minutes to remove insoluble material. The protein concentration in the supernatants was assayed using the Bradford dye method [14], using BSA as a standard curve and Bradford reagent (Bio-Agency Lab, São Paulo, SP, BRA). For sodium dodecyl sulfate gel electrophoresis and Western blot analysis, the samples were treated with a Laemmli sample buffer containing dithiothreitol. After heating to 95°C for 5 minutes, the proteins were separated by electrophoresis (70 μg protein per lane, 10% gels) and, afterward, transferred to nitrocellulose membranes. The nitrocellulose filters were treated overnight with a blocking buffer (5% nonfat dried milk, 10 mmol/L Tris, 150 mmol/L NaCl, and 0.02% Tween 20) and were subsequently incubated with a rabbit polyclonal antibody against PLCβ_2 (1:500; cat. [H-255] sc 9018) or PKAα (1:500; cat. [C-20] sc 903), mouse monoclonal antibody to PKCα (1:1000; cat. [H-7] sc 8393), or rabbit polyclonal antibody to phospho (p)-PKCα/βII (Thr638/641) (1:1000; cat. 9375S) at 4°C. Visualization of specific protein bands was made by incubating the membranes for 2 hours with a peroxidase-conjugated secondary antibody (1:10 000; Zymed Laboratories, San Francisco, CA), followed by detection with enhanced chemiluminescence reagents (Pierce Biotechnology, Rockford, IL) and exposure to x-ray film (Kodak, AM, Brazil). The band intensities were quantified by optical densitometry (Scion Image; Scion, Frederick, MD). After assaying the target proteins, Western blotting was repeated using rabbit polyclonal antibody to actin (1:1000; cat. [H-196]: sc-7210), used as an internal control for total proteins and for pPKC, results were expressed as a ratio of pPKCα/βII:PKCα expression. All primary antibodies used were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) with the exception of the pPKCα/βII antibody from Cell Signaling (Beverly, MA).

2.7. Statistical analysis

Results are presented as means ± SEM for the number of determinations (n) indicated. Statistical analyses were carried out using unpaired Student *t* test; the level of significance was set at $P < .05$, and analyses were performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA).

3. Results

3.1. Animal features

Low-fat chow–fed female and male mice aged 20 weeks, LDLR$^{-/-}$ mice, and WT mice were used in this study. As previously reported [12], LDLR$^{-/-}$ mice showed higher glycemia, hypoinsulinemia, and increased total CHOL and TG plasma levels when compared with WT mice ($P < .02$, $P < .005$, and $P < .0001$, respectively), whereas NEFA plasma concentrations were similar to those of WT (Table 1).

Table 1 – Fasting plasma glucose, insulin, CHOL, TG, and NEFA concentrations in LDLR$^{-/-}$ and WT mice

	WT	LDLR$^{-/-}$
Glucose (mg/dL)	67 ± 3	75 ± 2 *
Insulin (ng/mL)	0.27 ± 0.03	0.17 ± 0.02 *
CHOL (mg/dL)	107 ± 3	284 ± 7 *
TG (mg/dL)	107 ± 8	188 ± 7 *
NEFA (mmol/L)	1.20 ± 0.06	1.19 ± 0.07

Data are means ± SEM (n = 13-22).
* $P < .05$ vs WT mice.

3.2. Insulin secretion induced by depolarizing agents

In the presence of subthreshold (2.8 mmol/L) (Fig. 1A) and postprandial glucose concentrations (11.1 mmol/L) (Fig. 1B), the insulin secretion rates induced by depolarizing agents such as 40 mmol/L KCl, 10 mmol/L L-arginine, and 100 μmol/L tolbutamide were lower in LDLR$^{-/-}$ than in WT islets ($P < .05$). At 2.8 mmol/L glucose, the insulin secretion was not different between groups (0.12 ± 0.01 and 0.11 ± 0.01 ng per islet per hour for LDLR$^{-/-}$ and WT islets, respectively; n = 25), whereas at 11.1 mmol/L glucose, insulin secretion was 50% lower in LDLR$^{-/-}$ islets than in WT islets (0.77 ± 0.04 and 1.52 ± 0.11 ng per islet per hour, respectively; n = 20-22; $P < .05$). In accordance with insulin secretion experiments, KCl induced a lower Ca^{2+} influx in LDLR$^{-/-}$ islets (Fig. 2B) when compared with WT islets (Fig. 2A). The amplitude and total cytoplasmatic Ca^{2+} levels in response to KCl were lower in LDLR$^{-/-}$ islets compared with WT islets ($P < .03$ and $P < .01$, Fig. 2C and 2D, respectively).

3.3. Carbachol and phorbol 12-myristate 13-acetate induced insulin secretion

Fig. 3A shows that, at 11.1 mmol/L glucose, increasing concentrations of carbachol (Cch) (1-100 μmol/L), which stimulates the formation of inositol-1,4,5-triphosphate plus diacylglycerol through activation of PLC, dose-dependently increased insulin secretion in both groups of islets. Insulin secretion observed at all concentrations of Cch in LDLR$^{-/-}$ were similar to those of WT islets. In addition, the presence of 100 nmol/L phorbol 12-myristate 13-acetate (PMA), which stimulates PKC, also fully recovered insulin secretion in LDLR$^{-/-}$ islets (Fig. 3B).

3.4. Forskolin and 3-isobutyl-1-methyl-xanthine induced secretion

Forskolin (10 μmol/L) and 3-isobutyl-1-methyl-xanthine (IBMX) (1 mmol/L), which increase cyclic adenosine monophosphate (cAMP) by adenylate cyclase stimulation or by phosphodiesterase inhibition (respectively), significantly stimulated secretion in both types of islets (Fig. 4). However, the insulin secretion stimulated by both agents was markedly lower in LDLR$^{-/-}$ compared with WT islets. It should be noted that the decreased insulin release observed in LDLR$^{-/-}$ islets was not due to any alteration in insulin synthesis or storage because islet insulin content was similar between

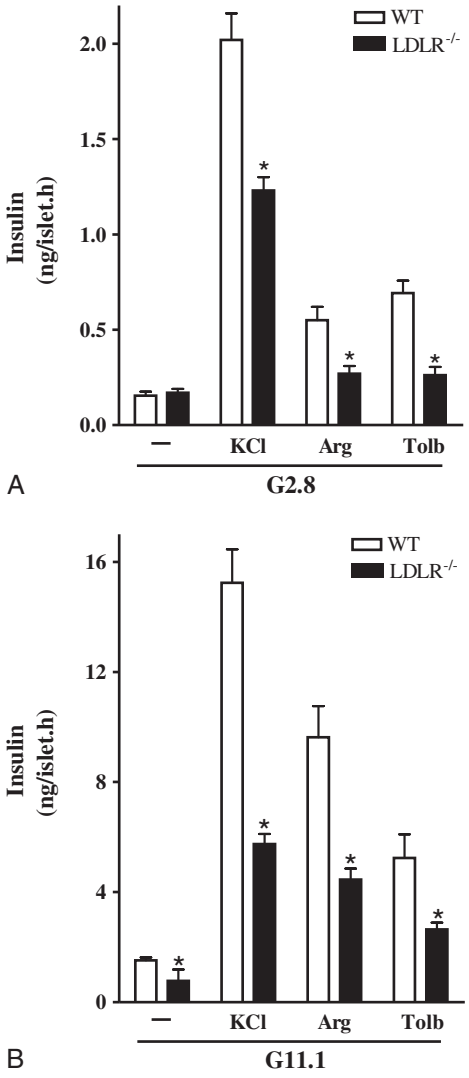

Fig. 1 – Insulin secretion induced by 40 mmol/L KCl, 10 mmol/L L-arginine, and 100 μmol/L tolbutamide (tolb) in isolated islets from LDLR$^{-/-}$ and WT mice in the presence of 2.8 (A) and 11.1 mmol/L glucose (B). Groups of 4 islets of similar sizes were incubated for 1 hour at 2.8 mmol/L (G2.8) or 11.1 mmol/L glucose (G11.1) without or with depolarizing agents. Each bar represents mean ± SEM of 10 to 25 groups of islets. *P < .05 related to respective control.

groups (54 ± 4 vs 51 ± 5 ng per islet for WT and LDLR$^{-/-}$ islet, respectively).

3.5. *PLCβ$_2$, PKCα, and PKAα protein expression*

Western blotting analysis shows that the expression of PLCβ_2 in LDLR$^{-/-}$ islets was 55% higher (n = 4, P < .05, Fig. 5A) and PKAα was 22% lower (n = 5, P < .05, Fig. 5D) when compared with WT islets, whereas the expression of PKCα (Fig. 5B) was similar between groups. When the ratio of pPKCα^{Thr638}/βII^{Thr641}/PKCα was measured, no difference between LDLR$^{-/-}$ and WT islets (Fig. 5C) was observed, showing that threonine residue phosphorylation, required for PKC catalytic competence [15], was not altered between the groups.

4. Discussion

In addition to hypercholesterolemia, LDLR$^{-/-}$ mice submitted to diabetogenic diets demonstrate increased adiposity, hypertriglyceridemia, hyperglycemia, and peripheral insulin resistance, indicating that the dyslipidemia induced by the LDLR mutation and unbalanced diets impairs glucose homeostasis [10,11].We recently observed that, in the absence of metabolic factors induced by a high-fat diet, LDLR$^{-/-}$ mice show normal whole-body insulin sensitivity but postprandial hyperglycemia, hypoinsulinemia, and glucose intolerance. Furthermore, we demonstrated that the main cause for impaired glucose homeostasis was a reduced islet insulin secretion in response to glucose [12]. Here, we investigated possible additional mechanisms involved in the lower secretory response of LDLR$^{-/-}$ mice islets.

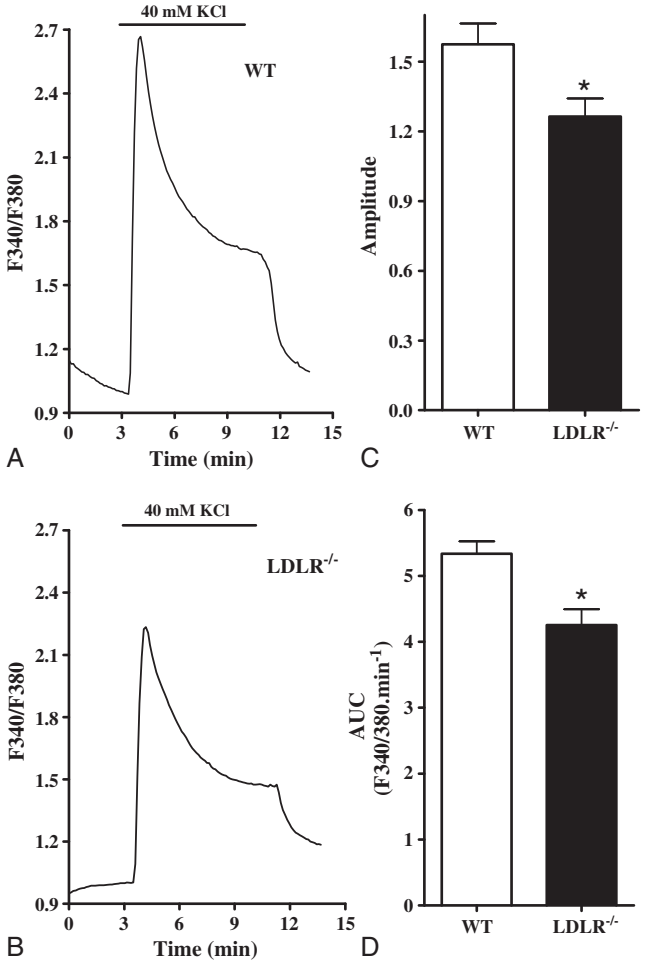

Fig. 2 – KCl (40 mmol/L) induced Ca^{2+} influx in isolated islets from LDLR$^{-/-}$ (A) and WT (B) mice. The experiments were performed in a perifusion system in the presence of 2.8 mmol/L glucose with or without 40 mmol/L KCl (horizontal lines). Values are the ratios of F340/F380 registered for each group. Data are means ± SEM obtained from 7 independent perifusion experiments. *P < .05 indicates significant difference vs WT.

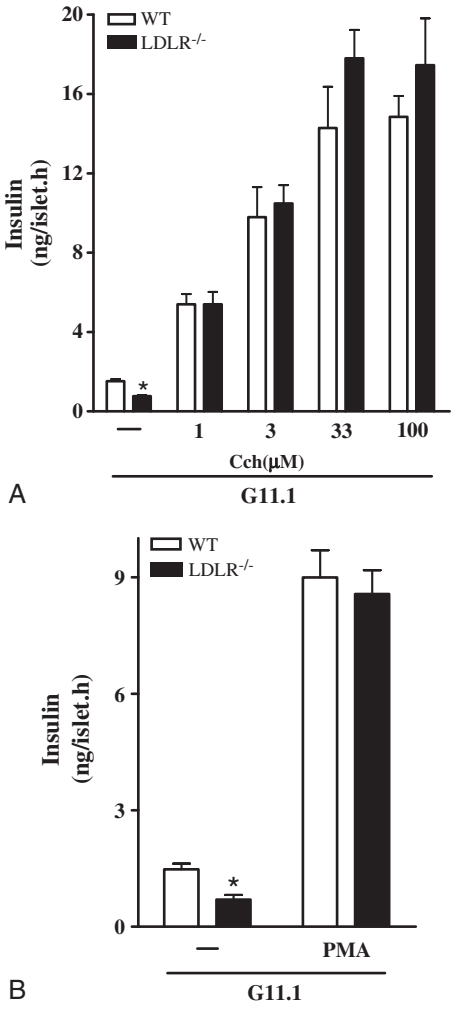

Fig. 3 – Insulin secretion induced by Cch (1-100 μmol/L) (A) and 100 nmol/L PMA (B) in isolated islets from LDLR$^{-/-}$ and WT mice. Groups of 4 islets of similar sizes were incubated for 1 hour at 11.1 mmol/L glucose (G11.1) with or without Cch or PMA. Each bar represents mean ± SEM of 15 groups of islets. *P < .05 related to respective control at the same concentrations of Cch and PMA.

First, we observed that depolarizing the β-cell membrane with high concentrations of KCl, L-arginine, and tolbutamide resulted in a lower insulin secretion in LDLR$^{-/-}$ compared with WT islets, both under basal and stimulatory glucose concentrations (Fig. 1). The lower secretion observed in LDLR$^{-/-}$ islets was not due to islet insulin and DNA content [12], but rather probably the consequence of other defects in the signal transduction pathways responsible for the secretory process.

Glucose stimulation of insulin secretion occurs by a synergistic interaction between at least 2 signaling pathways. One is the adenosine triphosphate (ATP)–sensitive K^+ channel–dependent pathway, where the closure of these channels depolarizes the β-cell membrane; provokes Ca^{2+} influx through voltage-gated channels; increases intracellular Ca^{2+} concentrations; and, ultimately, activates the extrusion of insulin granules. Our evidence suggests that the latter mechanism was impaired in LDLR$^{-/-}$ group because decreased insulin secretion in response to depolarizing agents occurred in LDLR$^{-/-}$ islets because of the lower Ca^{2+} influx (Figs. 1 and 2). This effect may be due to an impaired depolarization process and/or L-type voltage-sensitive Ca^{2+} channel activation or expression in the LDLR$^{-/-}$ group. It is known that membrane lipid composition may influence β-cell ion flux and channel activation [16]. We previously reported that LDLR$^{-/-}$ islets showed high CHOL levels and that plasma membrane CHOL depletion restored glucose-induced insulin release in LDLR$^{-/-}$ islets [12]. In addition, lower insulin secretion was observed in rodents that showed decreased or deletion of the α1.2 subunit of the L-type channel levels [17,18].

In the second pathway, glucose-stimulated insulin release is an ATP-sensitive K^+ channel–independent mechanism. In the former, glucose still increases insulin secretion without affecting the membrane potential (also known as the *Ca^{2+}-dependent pathway*) [19]. Because glucose metabolism was reduced in LDLR$^{-/-}$ islets by 30% [12], it may be postulated that the lower insulin secretion in LDLR$^{-/-}$ islets, which was not corrected by additional membrane depolarization (high KCl, L-arginine, and tolbutamide; Fig. 1B), may be to the consequence of alterations in the ATP-sensitive K^+ channel–independent pathway [20-22].

Activation of PKC is important for the phosphorylation of many proteins associated with the exocytotic process in β-cells [23]. Thus, we investigated the insulin secretory response to 11.1 mmol/L glucose in the presence of different concentrations of Cch, a muscarinic receptor agonist that enhances glucose-stimulated insulin secretion by activating the PLC/PKC pathway [24]. Carbachol restored the insulin secretory competence in LDLR$^{-/-}$ islets, reaching an even higher degree than that of WT islets (Fig. 2). This effect correlates well with a higher protein expression of PLCβ_2 in

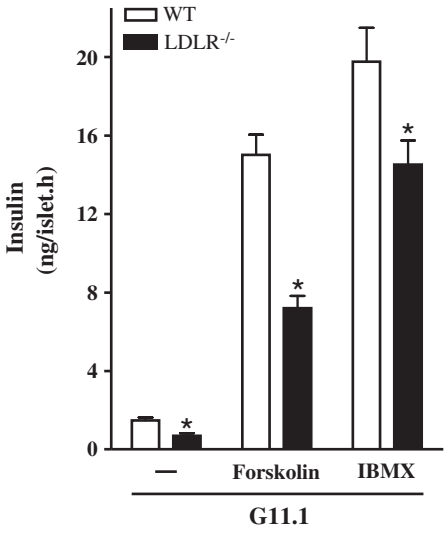

Fig. 4 – Insulin secretion induced by 10 μmol/L forskolin and 1 mmol/L IBMX in islets from LDLR$^{-/-}$ mice and WT mice. Groups of 4 islets of similar sizes were incubated for 1 hour at 11.1 mmol/L glucose (G11.1) with or without forskolin or IBMX. Each bar represents mean ± SEM of 14 to 15 groups of islets. *P < .05 related to respective control.

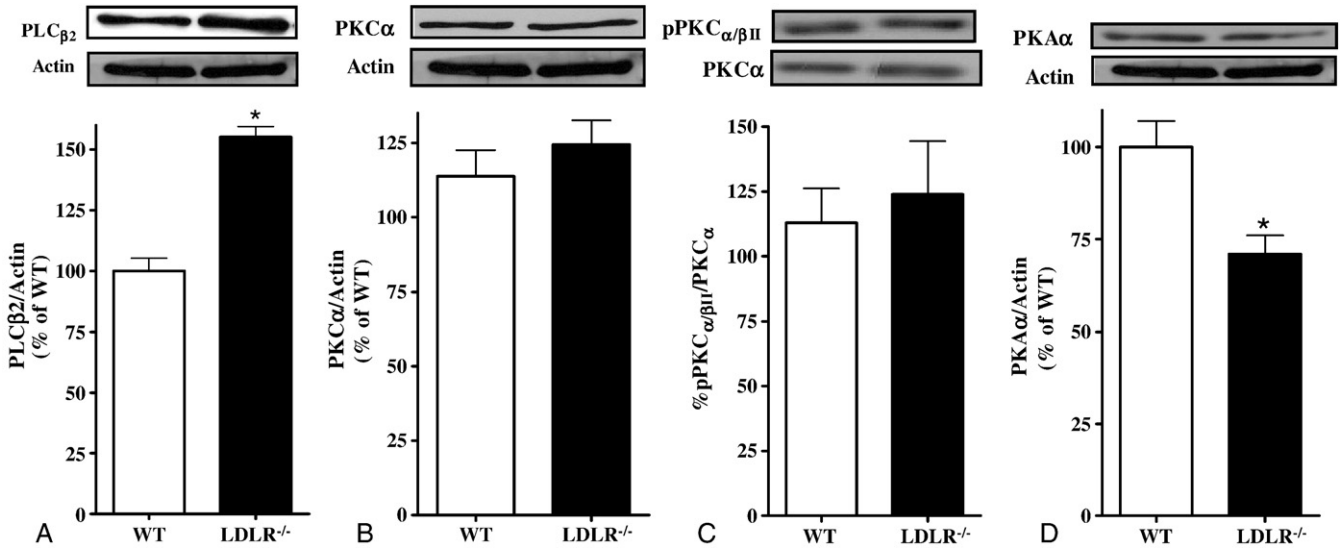

Fig. 5 – PLCβ_2 (A), PKCα (B), pPKCα/βII:PKCα (C), and PKAα (D) protein expression in isolated islets from LDLR$^{-/-}$ and WT mice. Protein extracts were processed for Western blotting. The bars represent the means ± SEM of the values, determined by optical densitometry. n = 3 to 5; *P < .05 vs WT.

LDLR$^{-/-}$ islets (Fig. 4A). Although the expression of PKCα was not altered in LDLR$^{-/-}$ islets, we also observed a full recovery of the insulin secretory response of these islets when PKC was directly stimulated by PMA. These findings suggest that the increased PLC expression may be a compensatory mechanism, which warrants normal in vivo insulin secretion at basal, nonstimulatory glucose concentrations; however, this is not enough to cope with the full response to postprandial levels of glucose.

The importance of the cAMP/PKA pathway for insulin secretion process has long been demonstrated [25]. Dyachok et al [26] monitored alterations in cAMP levels in β-cells by observing the dissociation of PKA catalytic from the regulatory subunits. These authors showed that glucose induces cAMP level oscillations, demonstrating that the cAMP/PKA pathway is important for glucose-induced insulin secretion. In addition, several hormones (incretins) use this pathway to potentiate glucose-stimulated insulin secretion [27-29]. These effects involve a series of reactions triggered by PKA, including the phosphorylation of vesicular and plasma membrane proteins, voltage-dependent channels, and transcription factors [23,30]. Protein kinase A activity seems to be specifically involved in the granule fusion reaction [30,31] because reduction of PKA expression diminishes the phosphorylation of proteins involved in the extrusion of insulin granules, such as snapin [32] and synaptosome-associated protein of 25 kd [33]. In support of this view, reduced glucose-induced insulin secretion in isolated islets and insulinoma cells is observed by inhibition of PKA [34] and lower PKAα expression [35,36]. Thus, the concomitant reduction of PKAα expression and insulin secretion observed in LDLR$^{-/-}$ mice islets (Fig. 4C) is in agreement with these observations.

In mice islets, PKA inhibitors selectively reduced the number of exocytotic events during the initial period of the first phase of glucose-induced exocytosis [31]. Protein kinase A inhibitors did not affect the glucose-induced increase in intracellular Ca^{2+} concentrations, and the exposure of islets to high glucose concentrations increased the duration of Ca^{2+}-dependent insulin exocytosis, an effect not mimicked by 2-deoxy-D-glucose, indicating that a glucose metabolite is necessary for the process [31]. One candidate is ATP [37], given that glucose rapidly increases its cytosolic concentrations [38]. The fast mode of Ca^{2+}-dependent insulin exocytosis is augmented by intracellular ATP concentration ($[ATP]_i$) in a concentration-dependent manner [39], and this effect seems to require both cAMP and PKA. Activation of PKA was also observed by increasing $[ATP]_i$ even in the absence of increased concentrations of cAMP [37]. In our experiments, both forskolin and IBMX, which increase [cAMP]i, failed to fully restore the insulin secretory competence in LDLR$^{-/-}$ islets (Fig. 3), indicating a lower availability of PKA for activating the secretion process (Fig. 4C). As stated before, we have previously observed a 30% reduction in the glucose metabolism in LDLR$^{-/-}$ islets, suggesting a possible reduction in $[ATP]_i$. Because ATP and PKA are important for the final steps of insulin granule extrusion, the association of these abnormalities may explain the reduced insulin secretion in the presence of glucose and other stimulators in LDLR$^{-/-}$ islets.

In conclusion, these data indicate that the lower insulin secretion in LDLR$^{-/-}$ mice islets may also be explained by decreased Ca^{2+} influx and PKA expression. The mechanism responsible for this abnormality in LDL receptor defective islets remains to be elucidated.

Acknowledgment

This study is part of the PhD Thesis of ML Bonfleur and was supported by grants from the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Conselho Nacional para o Desenvolvimento Científico e Tecnológico (CNPq), Instituto Nacional de Obesidade e Diabetes (CNPq/FAPESP), and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior

(CAPES). We are grateful to Léscio D Teixeira for animal care and to Dr Nicola Conran for English editing.

REFERENCES

[1] Fujimoto WY. Background and recruitment data for the U.S. Diabetes Prevention Program. Diabetes Care 2000;23:B11–B13.
[2] Wilson PW. Diabetes mellitus and coronary heart disease. Am J Kidney Dis 1998;32:S89-100.
[3] Haffner SM. Management of dyslipidemia in adults with diabetes. Diabetes Care 1998;21:160-78.
[4] Ginsberg HN, Zhang YL, Hernandez-Ono A. Regulation of plasma triglycerides in insulin resistance and diabetes. Arch Med Res 2005;36:232-40.
[5] Ginsberg HN. Lipoprotein physiology in nondiabetic and diabetic states. Relationship to atherogenesis. Diabetes Care 1991;14:839-55.
[6] Caparevic Z, Kostic N, Ilic S, et al. Oxidized LDL and C-reactive protein as markers for detection of accelerated atherosclerosis in type 2 diabetes. Med Pregl 2006;59:160-4.
[7] Cohen MP, Jin Y, Lautenslager GT. Increased plasma glycated low-density lipoprotein concentrations in diabetes: a marker of atherogenic risk. Diabetes Technol Ther 2004;6: 348-56.
[8] Breslow JL. Mouse models of atherosclerosis. Science 1996;272:685-8.
[9] Ishibashi S, Brown MS, Goldstein JL, et al. Hypercholesterolemia in low density lipoprotein receptor knockout mice and its reversal by adenovirus-mediated gene delivery. J Clin Invest 1993;92:883-93.
[10] Merat S, Casanada F, Sutphin M, et al. Western-type diets induce insulin resistance and hyperinsulinemia in LDL receptor-deficient mice but do not increase aortic atherosclerosis compared with normoinsulinemic mice in which similar plasma cholesterol levels are achieved by a fructose-rich diet. Arterioscler Thromb Vasc Biol 1999;19: 1223-30.
[11] Schreyer SA, Vick C, Lystig TC, et al. LDL receptor but not apolipoprotein E deficiency increases diet-induced obesity and diabetes in mice. Am J Physiol Endocrinol Metab 2002;282: E207–E214.
[12] Bonfleur ML, Vanzela EC, Ribeiro RA, et al. Primary hypercholesterolaemia impairs glucose homeostasis and insulin secretion in low-density lipoprotein receptor knockout mice independently of high-fat diet and obesity. Biochim Biophys Acta 2010;1801:183-90.
[13] Scott AM, Atwater I, Rojas E. A method for the simultaneous measurement of insulin release and B cell membrane potential in single mouse islets of Langerhans. Diabetologia 1981;21:470-5.
[14] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72: 248-54.
[15] Newton AC. Protein kinase C: structural and spatial regulation by phosphorylation, cofactors, and macromolecular interactions. Chem Rev 2001;101:2353-64.
[16] Xia F, Gao X, Kwan E, et al. Disruption of pancreatic beta-cell lipid rafts modifies Kv2.1 channel gating and insulin exocytosis. J Biol Chem 2004;279:24685-91.
[17] Schulla V, Renstrom E, Feil R, et al. Impaired insulin secretion and glucose tolerance in beta cell-selective Ca(v)1.2 Ca2+ channel null mice. Embo J 2003;22:3844-54.
[18] Iwashima Y, Abiko A, Ushikubi F, et al. Downregulation of the voltage-dependent calcium channel (VDCC) beta-subunit mRNAs in pancreatic islets of type 2 diabetic rats. Biochem Biophys Res Commun 2001;280:923-32.
[19] Straub SG, Sharp GW. Glucose-stimulated signaling pathways in biphasic insulin secretion. Diabetes Metab Res Rev 2002;18:451-63.
[20] Gembal M, Detimary P, Gilon P, et al. Mechanisms by which glucose can control insulin release independently from its action on adenosine triphosphate-sensitive K+ channels in mouse B cells. J Clin Invest 1993;91:871-80.
[21] Gembal M, Gilon P, Henquin JC. Evidence that glucose can control insulin release independently from its action on ATP-sensitive K+ channels in mouse B cells. J Clin Invest 1992;89:1288-95.
[22] Sato Y, Aizawa T, Komatsu M, et al. Dual functional role of membrane depolarization/Ca2+ influx in rat pancreatic B-cell. Diabetes 1992;41:438-43.
[23] Jones PM, Persaud SJ. Protein kinases, protein phosphorylation, and the regulation of insulin secretion from pancreatic beta-cells. Endocr Rev 1998;19:429-61.
[24] Boschero AC, Szpak-Glasman M, Carneiro EM, et al. Oxotremorine-m potentiation of glucose-induced insulin release from rat islets involves M3 muscarinic receptors. Am J Physiol 1995;268:E336–E342.
[25] Malaisse WJ, Pipeleers DG, Levy J. The stimulus-secretion coupling of glucose-induced insulin release. XVI. A glucose-like and calcium-independent effect of cyclic AMP. Biochim Biophys Acta 1974;362:121-8.
[26] Dyachok O, Idevall-Hagren O, Sagetorp J, et al. Glucose-induced cyclic AMP oscillations regulate pulsatile insulin secretion. Cell Metab 2008;8:26-37.
[27] Thorens B. GLP-1 and the control of insulin secretion. Journ Annu Diabetol Hotel Dieu 1994:33-46.
[28] Szecowka J, Grill V, Sandberg E, et al. Effect of GIP on the secretion of insulin and somatostatin and the accumulation of cyclic AMP in vitro in the rat. Acta Endocrinol (Copenh) 1982;99:416-21.
[29] Huypens P, Ling Z, Pipeleers D, et al. Glucagon receptors on human islet cells contribute to glucose competence of insulin release. Diabetologia 2000;43:1012-9.
[30] Seino S, Shibasaki T. PKA-dependent and PKA-independent pathways for cAMP-regulated exocytosis. Physiol Rev 2005;85: 1303-42.
[31] Hatakeyama H, Kishimoto T, Nemoto T, et al. Rapid glucose sensing by protein kinase A for insulin exocytosis in mouse pancreatic islets. J Physiol 2006;570:271-82.
[32] Chheda MG, Ashery U, Thakur P, et al. Phosphorylation of Snapin by PKA modulates its interaction with the SNARE complex. Nat Cell Biol 2001;3:331-8.
[33] Nagy G, Reim K, Matti U, et al. Regulation of releasable vesicle pool sizes by protein kinase A-dependent phosphorylation of SNAP-25. Neuron 2004;41:417-29.
[34] Wang X, Zhou J, Doyle ME, et al. Glucagon-like peptide–1 causes pancreatic duodenal homeobox-1 protein translocation from the cytoplasm to the nucleus of pancreatic beta-cells by a cyclic adenosine monophosphate/protein kinase A-dependent mechanism. Endocrinology 2001;142: 1820-7.
[35] Ferreira F, Barbosa HC, Stoppiglia LF, et al. Decreased insulin secretion in islets from rats fed a low protein diet is associated with a reduced PKAalpha expression. J Nutr 2004;134:63-7.
[36] Milanski M, Arantes VC, Ferreira F, et al. Low-protein diets reduce PKAalpha expression in islets from pregnant rats. J Nutr 2005;135:1873-8.
[37] Takahashi N, Kadowaki T, Yazaki Y, et al. Post-priming actions of ATP on Ca2+-dependent exocytosis in pancreatic beta cells. Proc Natl Acad Sci U S A 1999;96:760-5.
[38] Henquin JC. Cellular mechanisms of the control of insulin secretion. Arch Int Physiol Biochim 1990;98:A61–A80.
[39] Kasai H, Suzuki T, Liu TT, et al. Fast and cAMP-sensitive mode of Ca(2+)-dependent exocytosis in pancreatic beta-cells. Diabetes 2002;51:S19–S24.