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"EFEITO DA HIPERCOLESTEROLEMIA GENÉTICA SOBRE A HOMEOSTASE GLICÊMICA E SECREÇÃO DE INSULINA EM CAMUNDONGOS *KNOCKOUT* PARA O RECEPTOR DE LDL ($LDLR^{-/-}$)"

Tese apresentada ao Instituto de Biologia para obtenção do Título de Doutor em Biologia Funcional e Molecular, na área de Fisiologia.

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SUMÁRIO

RESUMO

Neste trabalho, investigamos se a hipercolesterolemia primária *per se*, independente de dieta rica em gordura, afeta a homeostase glicêmica e a secreção de insulina estimulada por vários secretagogos em animais *knockout* para o receptor de LDL ($LDLR^{-/-}$). Além disso, investigamos os possíveis mecanismos envolvidos na liberação deste hormôni**o** neste modelo animal. Podemos resumir nossos achados da seguinte maneira: camundongos $LDLR^{-/-}$ apresentam hiperglicemia pós-prandial, hipoinsulinemia, intolerância à glicose e sensibilidade periférica à insulina normal. Nós demonstramos que, as alterações na homeostase glicêmica ocorrem em parte, por uma diminuição da sensibilidade das ilhotas à glicose. A secreção de insulina é normal na presença de baixa concentração de glicose, entretanto na presença de 11,1 mmol/l, as ilhotas de animais $LDLR^{-/-}$ liberam menos insulina que as ilhotas controles. A secreção de insulina estimulada por outros secretagogos metabolizáveis (leucina e KIC) também está reduzida nas ilhotas dos animais *knockout*. O conteúdo total de insulina e DNA são similares entre os grupos, sugerindo que as alterações na secreção de insulina não ocorrem devido a diferenças no tamanho e/ou número de células β. Observamos uma redução na primeira e segunda fase de secreção de insulina estimulada por 11,1 mmol/l de glicose. A oxidação da glicose está reduzida, enquanto a metabolização da leucina está aumentada. Quando adicionamos agentes despolarizantes (KCl, Arginina e Tolbutamida), observamos uma redução da secreção de insulina tanto em concentrações basais quanto estimulatórias de glicose. Na presença de 11,1 mmol/l de glicose e carbacol (agonista colinérgico) ou PMA (ativador da proteína-quinase C), a secreção de insulina foi semelhante entre os grupos $LDLR^{-/-}$ e controles. Entretanto, quando estimulamos a secreção com forskolin ou IBMX, que aumentam os níveis de AMPc, observamos redução na liberação de insulina pelas ilhotas dos animais $LDLR^{-/-}$ em comparação com os controles. A expressão protéica da fosfolipase C (PLCβ2) está aumentada enquanto que a expressão da proteína-quinase A (PKA) está reduzida nas ilhotas dos animais $LDLR^{-/-}$. Assim, observamos que camundongos $LDLR^{-/-}$ apresentam alterações na homeostase glicêmica independente de dieta rica em gordura, provocada por redução na secreção de insulina devido, em parte à redução do metabolismo da glicose, bem como, redução na expressão da PKA.

ABSTRACT

In this work, we investigated whether primary hyperlipidemia *per se*, independently of a high-fat diet, affects glycemia and insulin secretion stimulated by several secretagogues in hypercholesterolemic low-density lipoprotein receptor knockout mice ($LDLR^{-/-}$). In addition, we investigated the possible mechanisms involved in the release of this hormone. We found that, besides higher total cholesterol and triglyceride plasma concentrations, glucose plasma levels were increased and insulin decreased in $LDLR^{-/-}$ compared to the wild type (WT) mice. $LDLR^{-/-}$ mice presented impaired glucose tolerance, but normal whole body insulin sensitivity. In addition, we also demonstrate that the main cause of the impaired glucose homeostasis is a reduced pancreatic islet insulin secretion ability following fuel secretagogue stimuli. $LDLR^{-/-}$ mice have impaired insulin secretion in response to glucose without alterations in the pancreatic total insulin and DNA contents. These findings support the idea that the decreased response to glucose cannot be explained by differences islet size or number of beta cells, but it is probably caused by a defect in the secretory process. Glucose oxidation was 30% lower and L-leucine oxidation 60% higher in $LDLR^{-/-}$ islets than in WT islets. 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 WT islets. Insulin secretion induced by the PKA activators, forskolin and IBMX, in the presence of 11.1 mmol/l glucose, was lower in $LDLR^{-/-}$ islets, and it was normalized in the presence of the PKC pathway activators, carbachol and PMA. Western blotting analysis showed that phospholipase C-β2 expression was increased and PKA-α decreased in $LDLR^{-/-}$ compared with WT islets. In conclusion, we demonstrate that genetic hypercholesterolemia, due to complete deficiency of LDLR, impairs the beta cell insulin secretion, leading to hyperglycemia without affecting body insulin sensitivity. The lower insulin secretion in $LDLR^{-/-}$ mice islets may be explained by reduced glucose metabolism and expression of PKA.

LISTA DE ABREVIATURAS

AGL	Ácidos graxos livres
ADP	Adenosina difosfato
AMPc	Adenosina monofosfato cíclico
ATP	Adenosina trifosfato
Apo	Apoliproteínas
CE	Ésteres de colesterol
CL	Colesterol livre
COL	Colesterol
DAC	Doenças cardiovasculares
DAG	Diacilglicerol
DM	Diabetes mellitus
HF	Hipercolesterolemia familiar
HDL	Lipoproteína de alta densidade
HMG-CoA	3-hidroxi-3-metil-glutaril coenzima A
IDL	Lipoproteína de densidade intermediária
IP3	Inositol 1,4,5-trifosfato
LCAT	Lecitina colesterol acil transferase
LDL	Lipoproteína de baixa densidade
LDLR	Receptor de LDL
$LDLR^{-/-}$	Camundongos *knockout* para o LDLR
LP	Lipoproteína
LPL	Lipoproteína lipase
LRP	*LDL receptor related protein*
PKA	Proteína quinase A
PKC	Proteína quinase C
PL	Fosfolípides
PLA_2	Fosfolipase A_2
PLC	Fosfolipase C
QM	Quilomícrons
TG	Triglicérides
VLDL	Lipoproteína de densidade muito baixa

I - APRESENTAÇÃO

Dislipidemia é a complicação mais comum na resistência à insulina e diabetes mellitus (DM), e pode ser exarcebada pela obesidade bem como por fatores ambientais, tais como dieta rica em gordura e sedentarismo (Avramoglu, Basciano et al. 2006). A dislipidemia diabética caracteriza-se por aumento na concentração plasmática de triglicerídeos (TG), redução das lipoproteínas (LP) de alta densidade (HDL) e alterações qualitativas das LP de baixa densidade (LDL), tornando-as mais aterogênicas (Ginsberg and Tuck 2001; Siqueira, Abdalla et al. 2006). Estas anormalidades nos lipídios plasmáticos e nas concentrações das LP são importantes para o aumento de morbidade e mortalidade provocado por doenças cardiovasculares (DAC) em indivíduos diabéticos e predispostos ao DM (Wilson, McGee et al. 1981; Haffner 1998). Embora existam poucos estudos sobre o papel das LP na fisiologia das células β pancreáticas, modificações nas LP observadas em DM podem contribuir para patogênese e progressão de disfunções nestas células.

Modelos animais que exibem alterações no metabolismo das LP são utilizados para elucidar os mecanismos pelos quais o metabolismo lipídico afeta o desenvolvimento do DM. Os efeitos deletérios de dietas ricas em gordura sobre a homeostase glicêmica e a predisposição ao DM têm sido demonstrados em diversos modelos animais (Eto, Yamashita et al. 2002; Poitout and Robertson 2002; Cerf 2007). Camundongos *knockout* para o receptor de LDL ($LDLR^{-/-}$), um modelo de hipercolesterolemia familiar (HF), quando submetidos à dieta rica em gordura, em adição à severa hiperlipidemia e aterosclerose, desenvolvem obesidade e diabetes, caracterizada por hiperglicemia, hiperinsulinemia e resistência periférica à insulina (Merat, Casanada et al. 1999).

Neste trabalho, investigamos se a hipercolesterolemia primária *per se*, independente de dieta rica em gordura, afeta a homeostase glicêmica e a secreção de insulina estimulada por vários secretagogos em animais $LDLR^{-/-}$. Além disso, investigamos os possíveis mecanismos envolvidos com a liberação de insulina neste modelo animal.

II - INTRODUÇÃO

METABOLISMO DAS LIPOPROTEÍNAS

O colesterol (COL) e os ésteres de colesterol (CE) assim como os TG e os fosfolípides (PL), provenientes da dieta ou sintetizados pelo próprio organismo necessitam de um sistema transportador para levá-los do tecido de origem para os tecidos nos quais eles serão armazenados ou consumidos. Assim, são transportados na circulação sanguínea complexados com proteínas denominadas de lipoproteínas (LP) e são captados pelas células em processos mediados por receptores ou transportadores.

As LP são complexos macromoleculares formados por um núcleo que contém combinações variadas de TG, CE e vitaminas lipossolúveis envolvido por uma monocamada anfipática de PL, colesteróis livre (CL) e proteínas específicas denominadas de apoliproteínas (apo), as quais mantêm a estabilidade estrutural da partícula e exercem um papel fundamental na regulação do metabolismo intravascular e destino das lipoproteínas (Brown, Kovanen et al. 1981).

Existem cinco classes de LP de acordo com sua densidade: os quilmícrons (QM), que transportam TG e COL exógenos do intestino para os tecidos; as LP de densidade baixa, intermediária e muito baixa, respectivamente LDL, IDL e VLDL, que transportam TG e COL endógenos (do fígado para os tecidos); e as LP de alta densidade (HDL), que transportam COL endógeno dos tecidos para o fígado.

Os QM são sintetizados no retículo endoplasmático das células epiteliais do intestino delgado e incorporam os lípides da dieta. Após sua formação, os QM são secretados no sistema linfático e entram na circulação sanguínea via veia cava superior. Os QM contém apoB-48 que é exclusiva e importante durante sua formação. Eles contém também apoE, apoCII e apoCIII. A apoCII ativa a lipoproteína lipase (LPL), enzima que é sintetizada e secretada na membrana basal das células endoteliais dos vasos sanguíneos de vários órgãos (Bisgaier and Glickman 1983). Esta hidrolisa os TG do núcleo dos QM e libera os ácidos graxos (AG) para os tecidos. Nesse processo, além de perder TG, os QM perdem também CL e PL para as HDL circulantes e ganham apoE e apoC, resultando em uma partícula denominada remanescente de QM, mais pobre em TG e mais rica em CE e apoE. Estes remanescentes são reconhecidos por receptores hepáticos de alta afinidade, os quais

promovem sua rápida captação pelos hepatócitos (Heath, Gahan et al. 2001). Estes receptores são denominados de receptores de remanescentes de QM, ou receptores E, ou ainda LRP (*LDL receptor-related protein*) (Herz, Hamann et al. 1988).

As VLDL são sintetizadas e secretadas pelo fígado e também contém TG, CE, CL e PL, além de apoB-100, apoAs, apoCs e apoE. Uma vez no plasma, as VLDL são hidrolisadas pela LPL reduzindo seu tamanho e formando os remanescentes de VLDL, as IDL. Grande parte das IDL é rapidamente captada via receptores hepáticos específicos (LRP) e o restante continua circulando, perdendo mais TG, PL e apoLPs, transformando-se então em LDL (Ginsberg and Tuck 2001), as quais praticamente não contém TG, são muito ricas em CE e preservam apenas a apoB100.

As HDL são sintetizadas no fígado, intestino delgado e em macrófagos como partículas pequenas discóides formadas basicamente de apo AI e PL podendo conter um pouco de CL. As HDL maduras contêm: apoA-I, apoC-I, apoC-II, entre outras apo, bem como a enzima lecitina colesterol aciltransferase (LCAT) que catalisa a formação de CE a partir da lecitina (fosfatidilcolina) e CL. A LCAT que atua na superfície das HDL nascentes converte a fosfatidilcolina e CL (provenientes tanto dos QM e VLDL como de tecidos extra-hepáticos) em CE. Este, por sua alta hidrofobicidade, passa então para o interior da HDL. As HDL maduras ricas em CE liberam este CE para o fígado, de onde será re-utilizado ou excretado como tal ou na forma de sais biliares na bile. A concentração plasmática elevada de HDL está associada à redução de risco de aterosclerose, visto que estas partículas também podem remover o CL dos tecidos extra-hepáticos e transportá-lo para o fígado por meio do chamado transporte reverso de colesterol, um eficiente mecanismo para descartar o excesso de colesterol dos tecidos. Além disso, as HDL tem ação anti-inflamatória, anti-oxidante e anti-trombótica (Stein and Stein 1999).

As LDL são formadas a partir da hidrólise dos TG das VLDL pela LPL e pela lipase hepática, formando inicialmente as IDL e subsequentemente as LDL. Estas partículas são ricas em CE e apoB100, sendo as principais transportadoras de colesterol sérico (cerca de 70% do colesterol total). Seu "turnover" é mais lento (2 a 3 dias) e seu *clearence* ocorre por meio da ligação das apoB100 com o receptor de LDL (LDLR), principalmente nos hepatócitos (75%), mas também nos outros tecidos extra-hepáticos (Siqueira, Abdalla et al. 2006). Dados epidemiológicos e estudos clínicos mostram que um aumento na concentração plasmática de

LDL é um fator de risco independente para o desenvolvimento de aterosclerose (Gordon and Rifkind 1989; Assmann 2001).

O LDLR é uma proteína transmembrana composta por 839 aminoácidos com uma função crítica na homeostase do colesterol celular e plasmático. Esses receptores foram primeiramente descritos por Brown & Goldstein (Brown and Goldstein 1986). O ligante fisiológico mais importante para o receptor é a LDL, mas também se liga a LP que contêm múltiplas cópias de apoE, tais como VLDL e IDL (Jeon and Blacklow 2005), uma vez que reconhece uma seqüência de aminoácidos idêndica nestas duas apoLP. A LDL liga-se com o LDLR na superfície celular, iniciando o processo de endocitose mediada por receptor. As vesículas endocíticas revestidas de clatrina fundem-se com endossomos precoces, que devido ao baixo pH, promovem a dissociação da LDL do receptor. O endossomo se funde com o lisossomo onde ocorre a hidrólise de CE a CL e hidrólise da apoB100. As membranas da vesícula contendo os LDLR se destacam e recirculam para membrana plasmática para internalizar mais LDL (Soccio and Breslow 2004).

Uma vez dentro da célula, o colesterol derivado das LP exerce várias ações regulatórias, incluindo a supressão da expressão da enzima que catalisa a etapa limitante da biossíntese do colesterol, a HMG-CoA redutase, e a supressão da transcrição do gene do receptor da LDL. Desta forma, as células se protegem de um aumento excessivo da concentração de colesterol. Por outro lado, na deprivação de colesterol, as células aumentam a expressão da HMG-CoA redutase estimulando a síntese de novo de colesterol e aumentam também a expressão dos receptores de LDL promovendo captação do colesterol exógeno. Os mecanismos moleculares envolvidos nesta fina regulação da concentração intracelular de colesterol foram bem elucidados pelos prêmios-Nobel Michael Brown e Joseph Goldstein (Brown and Goldstein 1997). Como consequência, a concentração de LDL no plasma varia em função do número de receptores de LDL nos tecidos, principalmente no fígado. Estes podem estar diminuídos por defeito genético ou por excesso de colesterol na dieta que aumenta o aporte de LP de origem intestinal (QM e remanescente) para o fígado.

Concentração elevada de LDL plasmática resulta em aumento da quantidade de LDL que se infiltra no espaço subendotelial. A LDL interage com elementos da matriz extracelular (ex: glicosaminoglicanos e colágenos) e fica retida por mais tempo neste microambiente, onde sofre alterações químicas tais como oxidação e proteólise. Esta LDL quimicamente

modificada estimula as células endoteliais a expressarem moléculas de adesão facilitando a adesão e transmigração de células linfomonocitárias circulantes para o espaço subendotelial. Os monócitos proliferam-se e diferenciam-se em macrófagos que captam as LDL modificadas via receptores *scavenger*. Diferentemente dos receptores de LDL, os receptores scavanger não são regulados e os macrófagos captam as LDL modificadas de maneira contínua transformando-se em *foam cells*. Com o tempo, as *foam cells* morrem e vão constituir o núcleo necrótico da lesão aterosclerótica avançada. Células musculares lisas também migram da camada adventícia para íntima, proliferam-se e eventualmente transformam-se em foam cells. O processo ocorre em progressão geométrica com a participação de um grande número de fatores de crescimento, citocinas e moléculas vasoregulatórias (Libby, Aikawa et al. 2000; Lusis 2000).

Defeitos genéticos na expressão do LDLR causam a hipercolesterolemia familiar (HF) em seres humanos (Hobbs, Brown et al. 1992), macacos rhesus (Scanu, Khalil et al. 1988), coelhos (Fan and Watanabe 2000) e em camundongos geneticamente modificados (Ishibashi, Brown et al. 1993; Ishibashi, Goldstein et al. 1994). A HF é uma desordem hereditária relativamente freqüente quando em heterozigoze, uma vez que as conseqüências da homozigoze levam a mortalidade precoce, geralmente na primeira ou segunda década de vida, antes da idade reprodutora. Mais de 600 mutações diferentes no gene LDLR humano já foram identificadas (Heath, Gahan et al. 2001) e a prevalência da forma heterozigótica é estimada em aproximadamente 1:500, embora possa ser maior em populações com efeito fundador (Yuan, Wang et al. 2006).

DISLIPIDEMIA E DIABETES MELLITUS

O DM é definido com uma síndrome de hiperglicemia crônica resultante da falta de produção de insulina e/ou de resistência à ação deste hormônio nos tecidos-alvo (Goldstein 2003). Em 1995, 4% da população mundial apresentava diagnóstico de DM e projeta-se um aumento para 5,4% em 2025, ou seja, a população de diabéticos de 135 milhões em 1995 passará para 300 milhões em 2025 (McDaniel, King et al. 1974).

O DM é classificado em dois tipos principais: a) DM insulino–dependente (DMID) ou tipo 1 e b) DM não-insulino-dependente (DMNID) ou tipo 2. A maioria (90-95%) dos indivíduos diagnosticados com esta síndrome apresenta o DM tipo 2. Vários estudos mostram

que o DM tipo 2 está associado com aumento de 3 a 4 vezes no risco de desenvolvimento de DAC (Wilson, McGee et al. 1981; Haffner 1998). Observa-se que 75% das mortes entre homens diabéticos e 57% entre as mulheres diabéticas são atribuídas a DAC (Schwartz 2006), e estas, são frequentemente mais severas e com prognóstico menos favorável em pacientes diabéticos do que não-diabéticos (Haffner, Lehto et al. 1998).

A relação entre DM e a ocorrência de eventos da DAC é positiva mas não-linear. Sugere-se que a hiperglicemia dos diabéticos está relacionada com o risco de desenvolvimento de DAC (Coutinho, Gerstein et al. 1999). Além disso, pacientes diabéticos apresentam anormalidades nas concentrações de lipoproteínas plasmáticas que, por si, aumentam o risco de DAC (Ginsberg and Tuck 2001; Taskinen 2003). A dislipidemia diabética caracteriza-se por aumento na concentração plasmática de TG resultante de uma maior secreção e menor *clearance* das VLDL, diminuição da HDL e modificações químicas nas LDL, como oxidação e glicação, apesar de níveis relativamente normais ou um pouco elevados desta partícula (Kesaniemi and Grundy 1982; Ballantyne, Grundy et al. 2000). Fatores secundários tais como, doenças renais, hipotiroidismo, consumo de álcool e desordens genéticas nas LP podem exacerbar a dislipidemia diabética e aumentar muito a concentração de TG (Schwartz 2006).

O tratamento da dislipidemia em pacientes diabéticos tem sido eficaz na redução de eventos cardiovasculares. Visto que 80% dos indivíduos diabéticos são ou foram obesos (Bray 1990), a redução do peso corporal é um dos fatores que melhora a dislipidemia nestes pacientes. Estudos com os índios PIMA (diabéticos) demonstraram que a redução do peso corporal diminui a síntese de VLDL, sem alterar a taxa de remoção destas LP e a atividade da LPL (Howard 1993). Quando uma dieta equilibrada associada com exercício físico não atinge o objetivo de reduzir o peso corpóreo, a farmacoterapia pode ser utilizada no tratamento da dislipidemia em pacientes diabéticos.

Agentes hipoglicemiantes utilizados no tratamento do DM tipo 2, tais como metformina e tiazolidinedionas, podem diminuir a concentração plasmática de TG para 10-15% e 15-25% respectivamente (Ginsberg, Plutzky et al. 1999). A utilização de inibidores da HMG-CoA redutase (enzima limitante no processo de síntese de colesterol), denominados de estatinas, reduzem significativamente os eventos cardiovasculares em pacientes com DM (Uusitupa, Niskanen et al. 1990; Pappan, Pan et al. 2005). A utilização de fibratos (fenofibrato e gemfibrozil) em pacientes DM tipo 2 diminui o TG plasmáticos em 20-35%, por diminuir a

Introdução

síntese de VLDL e aumentar a atividade da LPL (Ginsberg, Zhang et al. 2005). Ainda, os fibratos causam aumento de HDL-colesterol em 10-20% por aumentarem a síntese da apoAI. Assim, o uso de fármacos pode auxiliar no tratamento das dislipidemias reduzindo as DAC em pacientes diabéticos.

A dislipidemia diabética é considerada uma conseqüência da resistência à ação da insulina, principalmente no tecido adiposo e fígado. A deficiência ou resistência à insulina promovem: 1- aumento da lipólise no tecido adiposo e maior aporte de AGL para o fígado, o que aumenta a síntese de TG e secreção de VLDL, 2- aumento da síntese de apoB no fígado e conseqüente aumento de secreção de VLDL, 3- redução da atividade da LPL responsável por redução do *clearance* da VLDL, 4- aumento da atividade da lípase hepática, que produz LDL menores e mais densas, portanto mais aterogênicas e 5- redução da HDL por aumento do *clearance* renal da apoAI (Ginsberg 2000). A hiperglicemia dos diabéticos agrava este quadro por promover a glicação das LDL e das HDL.

Embora esteja bem claro o papel agravante da dislipidemia secundária ao diabetes, a pergunta que fazemos em nossa presente investigação é se a dislipidemia primária (genética) poderia ser causa ou pelo menos um fator predisponente ao DM.

SECREÇÃO E AÇÃO DA INSULINA

A insulina é sintetizada e secretada pelas células β pancreáticas. Sua secreção é ajustada continuamente, de acordo com as flutuações dos níveis de nutrientes circulantes, em especial, a glicose. A secreção também é modulada, direta ou indiretamente, por hormônios, neurotransmissores e agentes farmacológicos. Esse controle multifatorial permite que as células β secretem insulina em quantidade e tempo adequados, regulando a concentração de nutrientes no sangue em diferentes situações fisiológicas, como jejum, refeição, exercício físico, gravidez, lactação entre outras (Boschero 1996).

A glicose é o regulador mais importante da secreção de insulina (Figura 1). Quando captada pelas células β pancreáticas, através de um transportador específico (GLUT-2) presente na membrana plasmática, é fosforilada à glicose-6-fosfato pela enzima glicoquinase e, posteriormente, metabolizada gerando ATP. Isso resulta em um aumento da relação ATP/ADP, que provoca o fechamento de um canal de K^+ sensível ao ATP, presente na membrana da célula β. A redução do efluxo de K^+ das células leva à despolarização da

Introdução

membrana, que por sua vez, provoca a abertura de canais de Ca^{2+} sensíveis à voltagem, resultando em um massivo influxo deste íon, aumentando sua concentração intracelular (Ahren, Sauerberg et al. 1999; Rutter 2001). Estudos também demonstram que a metabolização da glicose nas células β estimula a adenilato ciclase, a qual induz a formação de AMPc que, por sua vez, estimula a proteína quinase A (PKA) (Figura 2). Além disso, a metabolização da glicose também estimula a hidrólise de fosfoinositídeos através da ativação da fosfolipase C (PLC) resultando na formação do inositol-1,4,5-trifosfato (IP_3) e diacilglicerol (DAG). O IP_3 se difunde pelo citoplasma e induz a liberação de Ca^{2+} de estoques intracelulares aumentando sua concentração citoplasmática, enquanto o DAG estimula a proteína quinase C (PKC) (Figura 3). Finalmente, a metabolização da glicose também estimula a formação do ácido araquidônico, através da ativação da fosfolipase A_2 (PLA_2). Todos estes eventos induzidos pela metabolização da glicose (aumento citoplasmático de Ca^{2+} e ativação da PKA, PKC e PLA_2) culminam com a exocitose de grânulos secretórios contendo insulina (Prentki and Matschinsky 1987; Ahren, Sauerberg et al. 1999; Rutter 2001).

A secreção de insulina também é regulada por outros nutrientes circulantes. Aminoácidos, como a arginina e leucina, estimulam a secreção de insulina. Além disso, ácidos graxos livres podem estimular ou inibir a secreção de insulina dependendo principalmente de concentração e tempo de exposição das células beta a estes nutrientes. Estas alterações induzidas por nutrientes são importantes para manutenção da homeostase glicêmica e lipidêmica. Quando presentes em altas concentrações no sangue, estes nutrientes estimulam a secreção de insulina, a qual por sua vez, permite um armazenamento periférico de glicose, aminoácidos e lipídios, para que as concentrações destes nutrientes voltem a valores normais na circulação. Hormônios intestinais também participam da regulação da liberação de insulina. O hormônio GLP-1 (*glucagon like peptide 1*) e o peptídeo gástrico insulinotrópico (GIP) são importantes durante a ingesta alimentar. Eles são liberados do intestino para circulação e potencializam a secreção de insulina. Outros hormônios, produzidos pelas ilhotas pancreáticas como o glucagon (estimulador) e a somatostatina (inibidor) exercem efeitos parácrinos sobre a secreção de insulina. Finalmente, o sistema nervoso autonômico possui um papel importante na modulação da secreção de insulina. O sistema nervoso parassimpático, via nervo vago, através de receptores muscarínicos na célula β, potencializa a secreção da insulina, enquanto o

sistema nervoso simpático, agindo por meio de receptores α-adrenérgicos, inibe a secreção de insulina (Ahren, Sauerberg et al. 1999).

De modo geral, a insulina, em altas concentrações no período de absorção alimentar, estimula processos anabólicos de armazenamento de reservas energéticas, isto é, promove a síntese de glicogênio, lipídios e proteínas, agindo principalmente nos músculos, no tecido adiposo e no fígado. Além disso, a insulina tem uma importante função de inibir a maior parte dos processos catabólicos do organismo.

A insulina exerce suas funções nas células a partir da sua ligação com um receptor da família das tirosinas quinases. Os efeitos da insulina se iniciam com sua ligação na subunidade extracelular (α) do receptor resultando na autofosforilação da subunidade β do receptor que, por sua vez, catalisa a fosforilação de resíduos de tirosinas de diversas proteínas intracelulares, incluindo os substratos do receptor da insulina (IRS 1-4), GAB-1, Shc, APS, $p60^{DOK}$, SIRPS e c-Cbl. Cada uma destas proteínas possui um sítio específico para o acoplamento com outros sinalizadores protéicos que possuem o domínio SH2 (assim denominados devido à homologia com o produto do oncogene *src*). Entre estas proteínas estão subunidades regulatórias de várias enzimas como a PI3-quinase, proteínas fosfatases, Syp, Grb2 e Ca^{2+}ATPase. As proteínas SH2 podem, diretamente e indiretamente, estimular uma cascata de fosforilações e desfosforilações, em resíduos de serina ou treonina de proteínas quinases, com conseqüente estimulação de enzimas-alvo da insulina (White and Kahn 1994; Patti and Kahn 1998).

A maioria dos pacientes com diabetes tipo 2 pode apresentar deficiência na ligação da insulina com seu receptor nos órgãos-alvo ou defeitos coletivamente chamados pós-receptor, por exemplo, defeitos na atividade quinase do receptor, bem como, nas vias de sinalização intracelular, também contribuem para resistência à insulina destes pacientes (Patti and Kahn 1998).

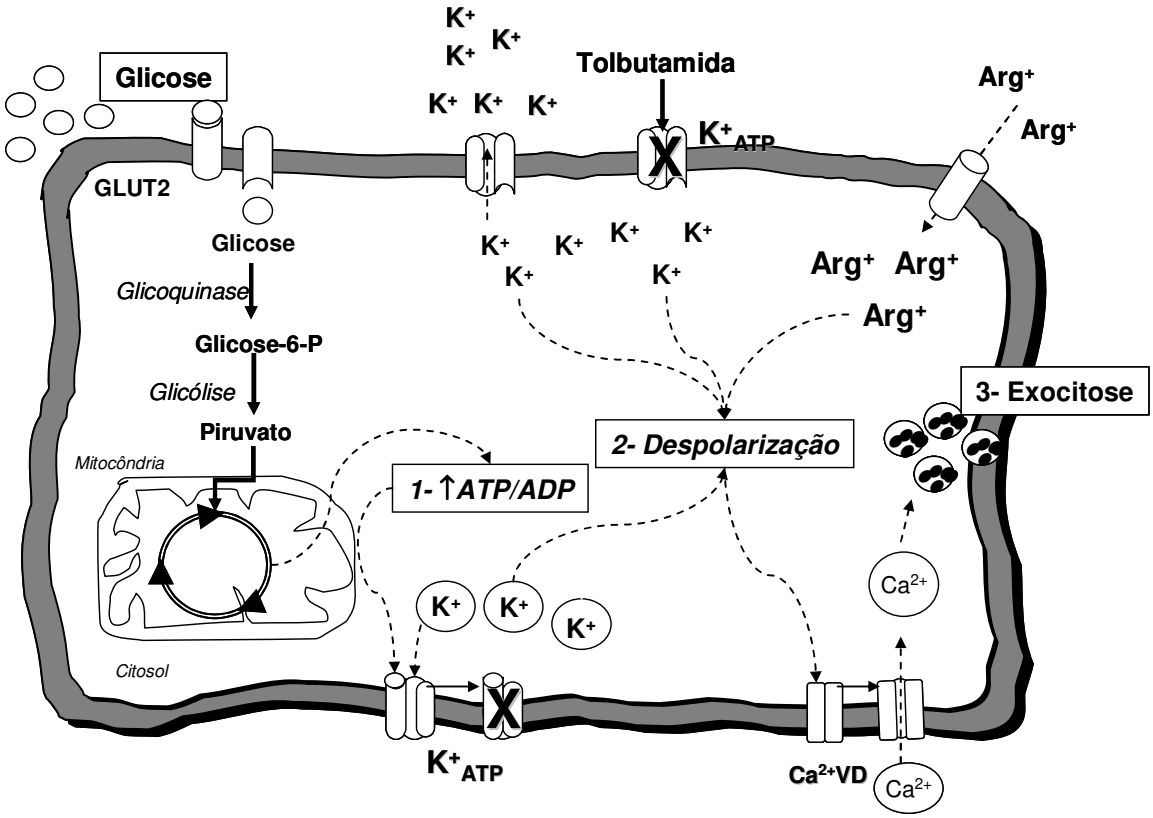

Figura 01 – Mecanismo de secreção de insulina estimulado pela glicose e por agentes despolarizantes. Quando ocorre um aumento da glicose plasmática, a célula beta pancreática capta este açúcar através de um transportador específico, o GLUT2. Como resultado da metabolização da glicose ocorre aumento da razão ATP/ADP da célula (1), fechamento dos canais de K^+ sensíveis ao ATP, despolarização da membrana (2) e exocitose (3) dos grânulos de insulina. Agentes despolarizantes, tais como KCl, tolbutamida (inibidor do canal de K^+ sensíveis ao ATP) e arginina provocam a despolarização da célula β e o processo de exocitose, independente do metabolismo da glicose.

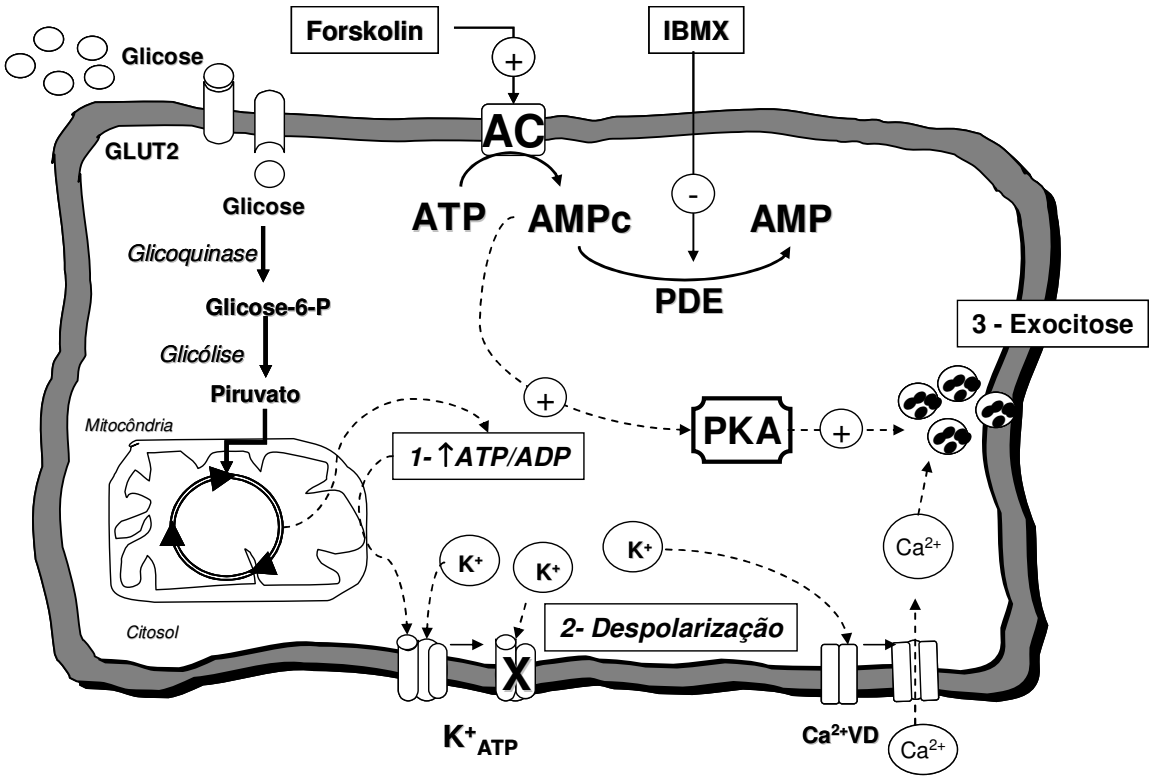

Figura 2 – Participação da via da proteína quinase A (PKA) na secreção de insulina pelas células β pancreáticas. A metabolização da glicose nas células β estimula a adenilato ciclase (AC), a qual induz a formação de AMPc que, por sua vez, estimula a PKA que age no processo de exocitose dos grânulos de insulina. Agentes que causam aumento de AMPc, como Forskolin (ativador da AC) ou IBMX (isobutil-metil-xantina, inibidor da fosfodisterase, PDE) são utilizados para evidenciar esta via de sinalização.

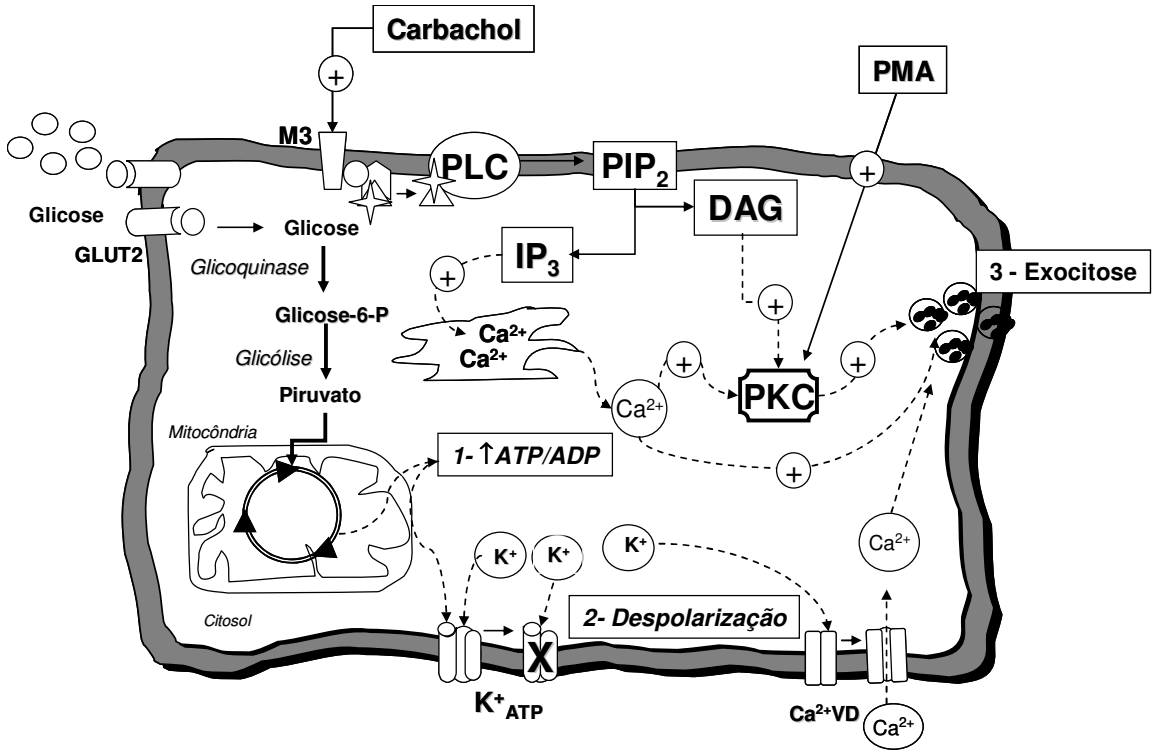

Figura 3 - Participação da proteína quinase C (PKC) na secreção de insulina pelas células β pancreáticas. A metabolização da glicose também estimula a hidrólise de fosfoinositídeos (PIP_2) de membrana através da ativação da fosfolipase C (PLC) resultando na formação do inositol-1,4,5-trifosfato (IP_3) e diacilglicerol (DAG). O IP_3 se difunde pelo citoplasma e induz a liberação de Ca^{2+} de estoques intracelulares aumentando sua concentração citoplasmática, enquanto o DAG juntamente com o Ca^{2+} estimula a PKC, a qual estimula a exocitose dos grânulos de insulina. Agonistas colinérgicos, como o carbacol, e ativadores diretos da PKC, como o PMA (*phorbol 12-myristate 13-acetate*), são utilizados para evidenciar esta via de sinalização.

O MODELO ANIMAL GENETICAMETE MODIFICADO

Animais geneticamente modificados para superexpressar (transgênicos) ou não-expressar (*knockout*) genes específicos envolvidos no transporte intravascular de lípides tem sido muito úteis para se investigar as repercussões metabólicas e patológicas de dislipidemias genéticas (Paigen, Plump et al. 1994; Breslow 1996). Foram criados e estudados transgênicos para quase todas as apoLP plasmáticas (Breslow 1996), para o LDLR (Hofmann, Russell et al. 1988), para a proteína de transferência de CE (Agellon, Walsh et al. 1991; Jiang, Agellon et al. 1992; Marotti, Castle et al. 1993) e outros genes relacionados à aterosclerose (Knowles and Maeda 2000).

Animais *knockout* para o LDLR (Ishibashi, Brown et al. 1993) apresentam o fenótipo de hipercolesterolemia familiar com elevados níveis plasmáticos de LDL-colesterol. Quando submetidos à dieta rica em gordura, desenvolvem aterosclerose e xantomatose severas (Ishibashi, Goldstein et al. 1994; Schreyer, Lystig et al. 2003), hipertrigliceridemia e obesidade (Li, Brown et al. 2000), hiperglicemia e resistência à insulina (Merat, Casanada et al. 1999). Usando este modelo *knockout* de receptor de LDL, recentemente foi mostrado que as mitocôndrias de vários tecidos destes animais produzem mais espécies reativas de oxigênio (EROS) comparadas às mitocôndrias controles, sugerindo que estas organelas podem estar envolvidas nas etapas mais precoces da aterogênese (Oliveira, Cosso et al. 2005). As mitocôndrias dos animais $LDLR^{-/-}$ produziam mais EROS e desenvolviam mais transição de permeabilidade de membrana, um processo que desencadeia morte celular, seja por necrose ou por apoptose (Kowaltowski, Castilho et al. 2001). O aumento de produção de EROS não foi relacionado a alterações de conteúdo de colesterol na membrana, fluidez da membrana ou atividade da superóxido dismutase mitocondrial, mas sim a um menor conteúdo de NADPH, que corresponde a principal fonte de equivalentes redutores para o sistema enzimático anti-oxidante mitocondrial. Foi proposto que a maior lipogênese e esteroidogênese verificadas nestes animais (para compensar a menor captação de LDL) seriam responsáveis pela menor disponibilidade de NADPH, levando à condição de estresse oxidativo nas células destes animais (Oliveira, Cosso et al. 2005).

III – JUSTIFICATIVA

A capacidade das ilhotas pancreáticas em responder a elevações na concentração plasmática de glicose com aumento na secreção de insulina é o evento principal na preservação da homeostase glicêmica e inibição da instalação do quadro de DM tipo II. Ela depende de uma fina regulação na maquinaria secretória das células β. Vários nutrientes, hormônios e neurotransmissores participam deste processo. Entre os nutrientes, observamos que os ácidos graxos podem estimular ou inibir a secreção de insulina dependendo do tempo de exposição das ilhotas a este nutriente. Entretanto, o papel das LP plasmáticas na função das células β pancreáticas tem sido pouco estudado.

Células β de humanos e ratos internalizam as partículas de LDL e VLDL a partir da ligação destas LP com o LDLR na superfície celular (Grupping, Cnop et al. 1997). A captação da LDL pelas células β pancreáticas e sua subseqüente oxidação pode ser prejudicial para estas células (Cnop, Hannaert et al. 2002). Em células HIT-T15, LDL oxidada reduz a expressão de RNAm da pré-pró-insulina, o conteúdo celular e a secreção de insulina estimulada pela glicose (Okajima, Kurihara et al. 2005). VLDL e LDL induzem apoptose de maneira dose-dependente em células βTC3 e ilhotas de camundongos com participação importante do LDLR, visto que, animais *knockout* para este receptor, tem uma menor taxa de apoptose quando expostas a estas LP (Roehrich, Mooser et al. 2003). Em células β pancreáticas de humanos, a captação de LDL e VLDL pode contribuir para acumulação intracelular de lipídios, sendo este processo mais evidente com o envelhecimento (Cnop, Grupping et al. 2000).

Levando-se em consideração: 1- as evidências acima expostas, 2- as anormalidades nas LP e lipídios plasmáticos observados no DM, 3- a alta freqüência de diabetes na população geral, 4- a relativa alta freqüência de mutações do receptor de LDL (em heterozigose) na população geral, e 5- a associação entre diabetes, distúrbios do metabolismo das LP e aumento no risco de desenvolvimento de DAC em pacientes diabéticos, nosso trabalho visa obter novos conhecimentos sobre o papel da hipercolesterolemia causada pela deficiência de receptor de LDL na homeostase glicêmica e secreção de insulina. Desta forma, camundongos *knockout* do receptor de LDL ($LDLR^{-/-}$) constituem um modelo muito útil para investigar a influência da hipercolesterolemia genética, *per se*, na ausência de dieta rica em gordura e de outros fatores predisponentes ao diabetes.

IV - OBJETIVOS

• OBJETIVO GERAL

Investigar se a hipercolesterolemia primária *per se*, independente de dieta rica em gordura, afeta a homeostase glicêmica e a secreção de insulina estimulada por vários secretagogos em camundongos *knockout* para o receptor de LDL ($LDLR^{-/-}$). Além disso, investigar os possíveis mecanismos envolvidos com a liberação deste hormônio neste modelo animal.

• OBJETIVOS ESPECÍFICOS

1 - Investigar o efeito da hipercolesterolemia genética independente de dieta rica em gordura sobre a tolerância à glicose, sensibilidade periférica à insulina, secreção de insulina estimulada pela glicose, leucina, ácido isocapróico e metabolismo da glicose e leucina nas ilhotas pancreáticas isoladas;

2 – Investigar os possíveis mecanismos envolvidos com as alterações na secreção de insulina, especificamente da via de sinalização da proteína quinase C e da proteína quinase A.

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VI - MÉTODOS, RESULTADOS E DISCUSSÃO

VI.1 – *Artigo 1 (Manuscrito submetido):* "Low-Density Lipoprotein Receptor Deficient Mice Present Impaired Glucose Homeostasis and Insulin Secretion Independently of High-Fat Diet"

Low-Density Lipoprotein Receptor Deficient Mice Present Impaired Glucose Homeostasis and Insulin Secretion Independently of High-Fat Diet

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ABSTRACT

Aims/hypothesis: We investigated whether primary hypercholesterolemia *per se*, independently of a high-fat diet, affects glycemia and insulin secretion in hypercholesterolemic low-density lipoprotein receptor knockout mice ($LDLR^{-/-}$). *Methods*: Glucose homeostasis was analyzed using OGTT and insulin tolerance test. Static and dynamic insulin secretion and fuel metabolism were also examined in isolated islets. *Results:* We found that, besides higher total cholesterol and triglyceride plasma concentrations, glucose plasma levels were increased and insulin decreased in $LDLR^{-/-}$ compared to the WT mice. $LDLR^{-/-}$ mice presented impaired glucose tolerance, but normal whole body insulin sensitivity. A dose-response curve of insulin secretion from islets stimulated by increasing concentrations of glucose (2.8-27 mM) was shifted to the right in $LDLR^{-/-}$ compared to WT islets. Half-maximal response doses (EC_{50}) were 13.3 ± 0.6 and 10.9 ± 0.3 mmol/l for $LDLR^{-/-}$ and WT islets, respectively ($P < 0.05$). Significant reductions in insulin secretion in response to L-leucine or 2-ketoisocaproic acid (KIC) were also observed in $LDLR^{-/-}$ compared to WT islets ($P < 0.05$). Total islet insulin and DNA content were similar in both groups. Finally, glucose oxidation was 30% lower and L-leucine oxidation 60% higher in $LDLR^{-/-}$ islets than in WT islets. *Conclusions/interpretation:* These results indicate that genetic hypercholesterolemia due to complete deficiency of LDL receptor impairs the beta cell insulin secretion, leading to hyperglycemia without affecting body insulin sensitivity. This represents an additional risk factor for diabetes and atherosclerosis in primary hypercholesterolemia, independently of dietary fat content**.**

Key words: glucose homeostasis; hypercholesterolemia; insulin secretion; LDL receptor

Abbreviations

CHOL	Cholesterol
FH	Familial hypercholesterolemia
IDL	Intermediate density lipoproteins
ITT	Insulin Tolerance Test
KC	Krebs cycle
KIC	2-ketoisocaproic acid
LDLR	LDL receptor
$LDLR^{-/-}$	LDL receptor knockout mice
TG	Triglycerides
WT	Wild-type mice

Introduction

The majority of plasma cholesterol is transported by LDL and 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 LDL 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 hypercholesterolemia (FH) in humans [3], rhesus monkeys [4], Watanabe heritable hyperlipidemic 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 dyslipidemia characterized by hypertriglyceridemia, increased hepatic secretion of 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 a high-fat diet (western type) for 16-18 weeks, in addition to severe hyperlipidemia and atherosclerosis, LDLR knockout mice ($LDLR^{-/-}$) develop obesity and diabetes, characterized by hyperglycemia, hyperinsulinemia and peripheral insulin resistance [17].

While diabetes induced dyslipidemia [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 poorly understood. Rat and human beta cells internalize LDL and VLDL through the high affinity LDLR [20]. The LDL uptake by pancreatic beta cells and its subsequent oxidation can be harmful to these cells [21]. Human purified VLDL and LDL induced a dose-dependent rate of apoptosis in insulin-secreting beta TC3 cell line and C57BL/6 mice islets with an important participation of LDLR and c-Jun N-terminal Kinase signaling pathway [22]. In human pancreatic beta cells, the LDL and VLDL uptake may contribute to the 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 in vitro cytotoxic effects through the formation of oxidized lipids [24]. In this regard, $LDLR^{-/-}$ mice constitute an interesting model for studying the association between primary hypercholesterolemia and putative alterations of insulin secretion.

In this study, we investigated whether primary hypercholesterolemia *per se*, without other metabolic confounding factors such as high fat diet, affects glycemia homeostasis and insulin secretion in hypercholesterolemic, chow fed LDL receptor knockout mice.

Material and methods

Animals LDLR$^{-/-}$ mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Control wild-type (WT) mice (C57BL/6 background) were obtained from the breeding colony at the Universidade Estadual de Campinas. Animal experiments were approved by the university's Committee for Ethics in Animal Experimentation (CEEA/UNICAMP). The mice had access to standard laboratory rodent chow diet (Nuvital CR1, Colombo, Paraná, Brazil) and water *ad libitum* and were housed at 22 ± 1ºC on a 12 h light/dark cycle. Both male and female mice of 16 - 20 weeks of age were used for the experiments.

Plasma Biochemical Analysis Blood samples were collected from either the retro-orbital plexus of anesthetized mice or from the tail tip. Total cholesterol (CHOL), triglycerides (TG) and non esterified fatty acids (NEFA) were measured in fresh plasma in the fasting state (12 hours) using standard commercial kits, according to the manufacturer's instructions (Boehringer Mannhein®, Germany; Merck®, Germany and Wako®, Germany, respectively). Glucose levels in the fasted and fed state were measured using a glucose analyzer (Accu-Chek Advantage, Roche Diagnostic®, Switzerland) and plasma insulin was measured by RIA using rat insulin standard.

OGTT and Insulin Tolerance Test (ITT) Mice were submitted to an OGTT at 16 weeks of age. Mice were fasted for 12 hours 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, 1
[25] 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.

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 [26]. After isolation, batches of 4 islets each were pre-incubated in 1 ml Krebs-Ringer bicarbonate buffer (KRBB) containing, in mmol/l: 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% BSA for 30 min at 37ºC. The islets were further incubated for 1 h in the presence of 2.8, 5.6, 8.3, 9.3, 11.1, 13, 16.7 or 27 mmol/l glucose. In a different set of experiments, insulin secretion was measured in response to glucose (2.8 mmol/l) in the absence and presence of L-Leucine (10 mmol/l) or 2-ketoisocaproic acid (KIC; 10 mmol/l). Total islet insulin content was extracted using the acid/ethanol solution followed by insulin RIA.

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 mmol/l) was present from the onset of the experiment and was elevated to 11.1 mmol/l 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.

Glucose and L-Leucine oxidation Batches of 20 islets each were incubated in KRBB containing 11.1 mmol /l glucose in the presence of D-[U^{14}-C] glucose or 2.8 mmol/l glucose plus 10 mmol/l L-Leucine in the presence of L-[U^{14}-C] leucine to measure $^{14}CO_2$ production [27, 28]. Sealed rubber lined capped vials containing opened microtubes with the islets were incubated for 2 h in a shaking water bath at 37ºC. Two-hundred µl of 1N HCl were injected through the rubber caps into the microtubes containing the islets to stop respiration and 200 µl of 1N 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.

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 and the level of significance was set at $P < 0.05$.

Results

Plasma lipids, glucose and insulin levels Total plasma CHOL and TG levels were 2.7- and 1.8-fold higher in LDLR$^{-/-}$ mice than in WT mice ($P < 0.05$), whereas NEFA was not different between the two groups (Tab. 1). Fasting glucose levels were slightly (4%), but significantly, higher in LDLR$^{-/-}$ than in WT 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 ($P < 0.05$) (Tab. 1).

Glucose and insulin tolerance tests In order to explain the higher glycemia and lower insulinemia 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 0 and 15 min. However, after 30 min, blood glucose levels were significantly increased in LDLR$^{-/-}$ mice compared with WT mice ($P < 0.05$). The elevated glucose levels in LDLR$^{-/-}$ persisted until 90 min. The area under the curve of the OGTT in LDLR$^{-/-}$ mice was significantly greater than that of WT mice (823.6 ± 16.0 *vs.* 621.3 ± 14.4 mg/dL.90 min for LDLR$^{-/-}$ and WT mice, respectively; $P < 0.05$). 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 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 hypercholesterolemic mice, we repeated the OGTT and ITT in aged (12 month old) mice. As shown in Fig. 1 C and D, LDLR$^{-/-}$ mice maintain the glucose intolerance but they still have normal insulin sensitivity at advanced age.

Glucose-stimulated insulin secretion by isolated pancreatic islet Since hyperglycemic 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 WT mice. Batches containing four islets each from both groups of mice were incubated with increasing glucose concentrations (2.8 – 27.0 mmol/l). 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 mmol/l, for LDLR$^{-/-}$ and WT islets, respectively ($P < 0.05$). At intermediate glucose concentrations (11.1 and 13.5 mmol/l), 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 insulinemia found in these mice.

Similar total islet insulin content (130.5 ± 13.1 and 105.6 ± 7.9 ng/islet for LDLR$^{-/-}$ and WT mice, respectively; n = 33-40), as well as DNA content (2.33 ± 0.3 and 2.63 ± 0.4 ng/islet for LDLR$^{-/-}$ and WT mice, respectively; n = 10-12), were found in both groups.

Dynamics of insulin secretion In order to confirm the previous experiment and analyze the insulin secretion in more detail, we determined the dynamics of glucose-induced insulin secretion by isolated islets along time using perfusion experiments (Fig. 3). In the presence of a non-stimulatory glucose concentration (2.8 mmol/l), 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 mmol/l 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 reached a peak of 2.9 ± 0.26 ng/50 islets.min^{-1} in LDLR$^{-/-}$ islets and of 7.9 ± 0.5 ng/50 islets.min^{-1} in WT islets; increases of 8.5- and 20- fold compared to their respective basal values. This first phase release was markedly lower in LDLR$^{-/-}$ than in WT islets ($P < 0.05$). During the second phase, the mean insulin secretion rate was also lower (60%) in LDLR$^{-/-}$ than in WT islets (0.52 ± 0.1 and 1.3 ± 0.3 ng/50 islets.min^{-1}, respectively; $P < 0.05$).

$_L$-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 mmol/l glucose in the absence and presence of 10 mmol/l $_L$-Leucine or 10 mmol/l KIC (Fig. 4). As in the previous experiments, the basal insulin release (2.8 mmol/l 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 insulin output compared to WT islets. The amount of insulin released to the medium represented 53% and 43% of that observed in WT islets, respectively ($P < 0.05$).

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 a 30% reduction in glucose oxidation when compared to WT islets (20 ± 1.6 and 29 ± 1.8 pmol/islet.2h^{-1}, respectively; n = 14-15, $P < 0.05$). 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.2h^{-1} for LDLR$^{-/-}$ and WT mice, respectively; n = 17-18, $P < 0.05$).

Discussion

LDLR deficient mice are a model of human FH in which LDLR are impaired or non functional [3]. These mice show a marked increase in plasma CHOL, almost exclusively in the IDL/LDL fraction [2]. In addition to hypercholesterolemia, when submitted to a western type diet, $LDLR^{-/-}$ mice develop hypertriglyceridemia and obesity [29], hyperglycemia and insulin resistance [17], and severe atherosclerosis [30]. However, none of these disturbances, except the hypercholesterolemia, 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 lack of LDLR in the absence of other metabolic factors induced by high fat or high carbohydrate unbalanced diets. Data show that, at the age of 16-20 weeks, male and female $LDLR^{-/-}$ mice fed a normal chow diet present post-prandial hyperglycemia and hypoinsulinemia, 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 ability following fuel secretagogue stimuli. Since the maintenance of islet functionality is important for glucose homeostasis, these data suggest that the complete deficiency of LDLR can contribute to the onset of diabetes.

Human and rat beta cells express LDLR that can internalize lipoproteins, mostly LDL and VLDL [20]. In addition, necrosis induced by prolonged exposure of rat beta cells to LDL was dependent on its 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 transcription. Apoptosis rate was dependent on the time of exposure and concentrations of VLDL and LDL [22]. However, it is well known that LDL can become rapidly oxidized when exposed to several cell types in

culture [31] 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 [32]. Indeed, in HIT-T15 cells, oxidized LDL reduced the expression of preproinsulin mRNA, the cellular insulin content, and the glucose stimulated insulin secretion [33].

Our *in vitro* results show that islets from $LDLR^{-/-}$ mice have impaired insulin secretion in response to glucose without alterations in the pancreatic total insulin and DNA contents. These findings support the idea that the decreased response to glucose cannot be explained by differences in the amount of insulin present in the pancreatic islets (islet size or number of beta cells), but it is probably caused by a defect in the secretory process. In addition, 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 distant reserve pool, whose release depends on fuel metabolism and Ca^{2+} concentrations but is also regulated by the cAMP and phosphoinositol pathways [34].

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 [35], 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 [36]. 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 [37]. 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. 4) [38, 39].

The reduced secretion in response to glucose in islets from LDLR$^{-/-}$ correlates well with the observed reduction in the glucose oxidation in these islets. Since these islets do not take up cholesterol from the external medium, it is possible that glucose complete oxidation to CO_2 is reduced as a consequence of an increased beta cell need for steroidogenesis and lipogenesis. Accordingly, hepatocytes from LDLR$^{-/-}$ mice showed a lower content of NADPH in the mitochondrial pool and their livers synthesized and secreted more TG and CHOL than the WT mice [40]. In addition, in pancreatic beta cells with accelerated lipogenesis induced by the overexpression of SREBP-1c, the utilization of acetyl-coA and glucose is increased with a concomitant reduction in the ATP synthesis and insulin secretion [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 [40], the increased L-leucine metabolism in pancreatic islets would increase the Krebs cycle substrates to fulfill the demand for de novo synthesis of cholesterol.

Therefore, we propose that an overall shift in fuel catabolism to support an augmented steroidogenesis instead of increasing ATP production may explain the reduction in fuel stimulated insulin secretion in LDLR$^{-/-}$ islet. Although not addressed in this study, we cannot discard the possibility of 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.

In conclusion, we demonstrate that genetic hypercholesterolemia, due to complete deficiency of LDLR, impairs the beta cell insulin secretion, leading to hyperglycemia without affecting body insulin sensitivity. This represents an additional risk factor for developing diabetes and atherosclerosis.

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Figure legends

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. Panel 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, 60 and 90 min for glucose measurements. Panel 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. *p<0.05.

Fig. 2 Glucose-stimulated insulin secretion in islets from WT (*open squares*) and LDLR$^{-/-}$ (*solid circles*) mice. Groups of 4 isolated islets were incubated for 60 min with different glucose concentrations, as indicated. Each point represents mean ± SEM of 12-20 groups of islets. *p<0.05.

Fig. 3 Effects of 11.1 mmol/l 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 mmol/l was present from the onset of the experiment and was increased to 11.1 mmol/l 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. *p<0.05.

Fig. 4 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 mmol/l) and low-glucose plus 10 mmol/l L-Leucine or KIC. The columns represent the means ± SEM (n=15-20). *p<0.05.

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

		WT		$LDLR^{-/-}$	n
CHOL (mg/dL)		105 ± 2.6	(21)	280 ± 6.4*	(22)
TG (mg/dL)		104 ± 4.9	(22)	188 ± 7.0*	(22)
NEFA (mmol/l)		1.2 ± 0.04	(20)	1.1 ± 0.05	(19)
Glucose (mg/dL)	Fasted	72 ± 2.0	(31)	75 ± 1.5*	(28)
	Fed	121 ± 2.1	(41)	145 ± 2.4*	(40)
Insulin (pg/mL)	Fasted	279 ± 28	(18)	156 ± 9*	(20)
	Fed	817 ± 56	(12)	523 ± 28*	(12)

Data are means ± SEM of n (in parenthesis). *p<0.05 *vs* WT.

Figure 1

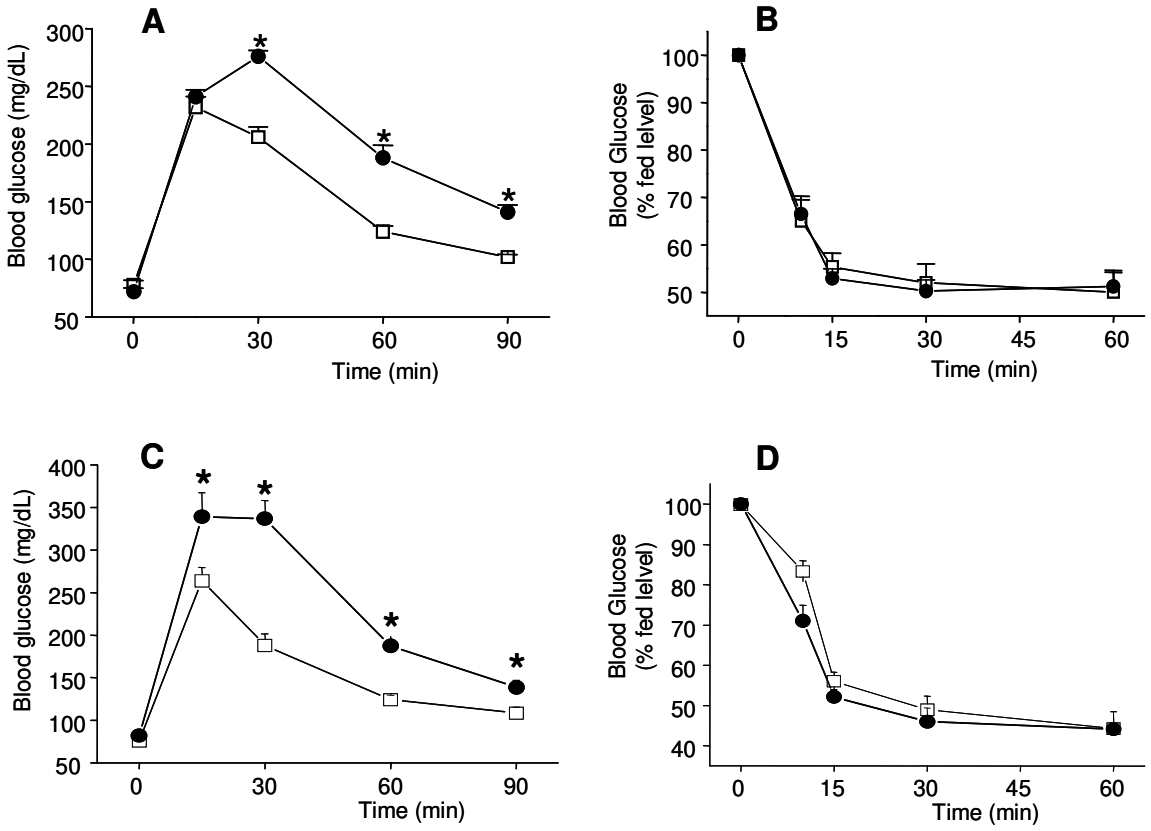

A
Blood glucose (mg/dL)
300
250
200
150
100
50
0
30
60
90
Time (min)
B
Blood Glucose
(% fed lelvel)
100
90
80
70
60
50
0
15
30
45
60
Time (min)
C
Blood glucose (mg/dL)
400
350
300
250
200
150
100
50
0
30
60
90
Time (min)
D
Blood Glucose
(% fed lelvel)
100
90
80
70
60
50
40
0
15
30
45
60
Time (min)

Figure 2

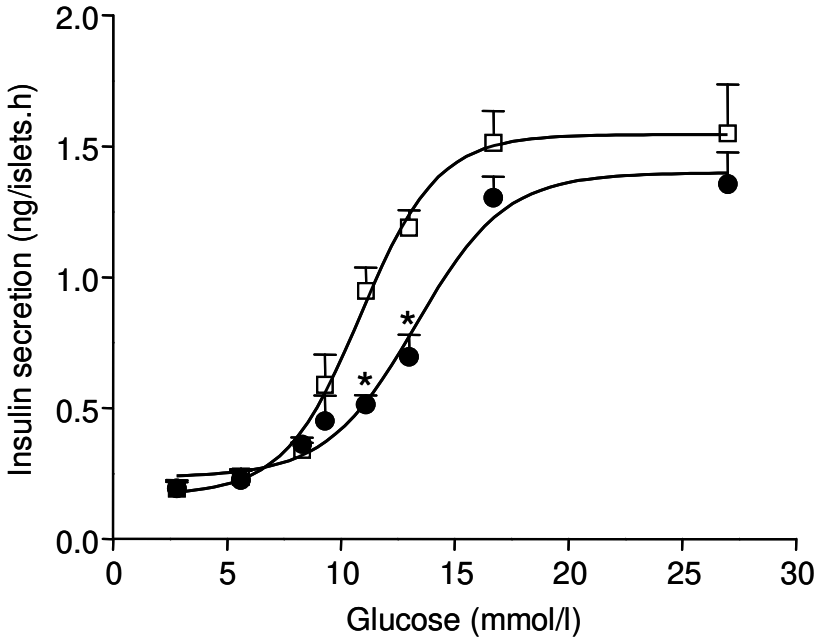

Insulin secretion (ng/islets.h)
2.0
1.5
1.0
0.5
0.0
0
5
10
15
20
25
30
Glucose (mmol/l)
*
*

Figure 3

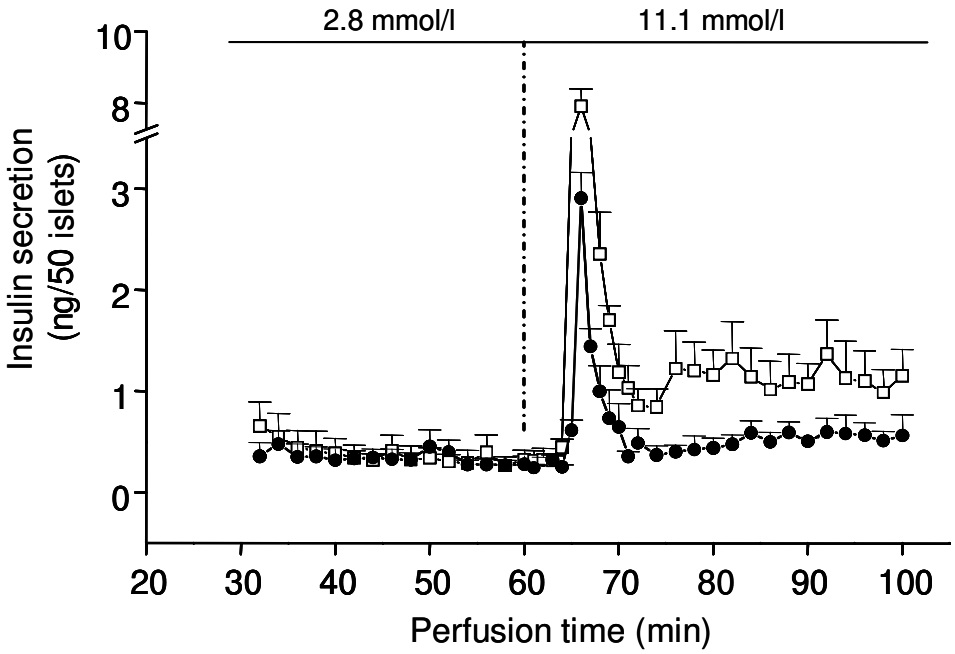
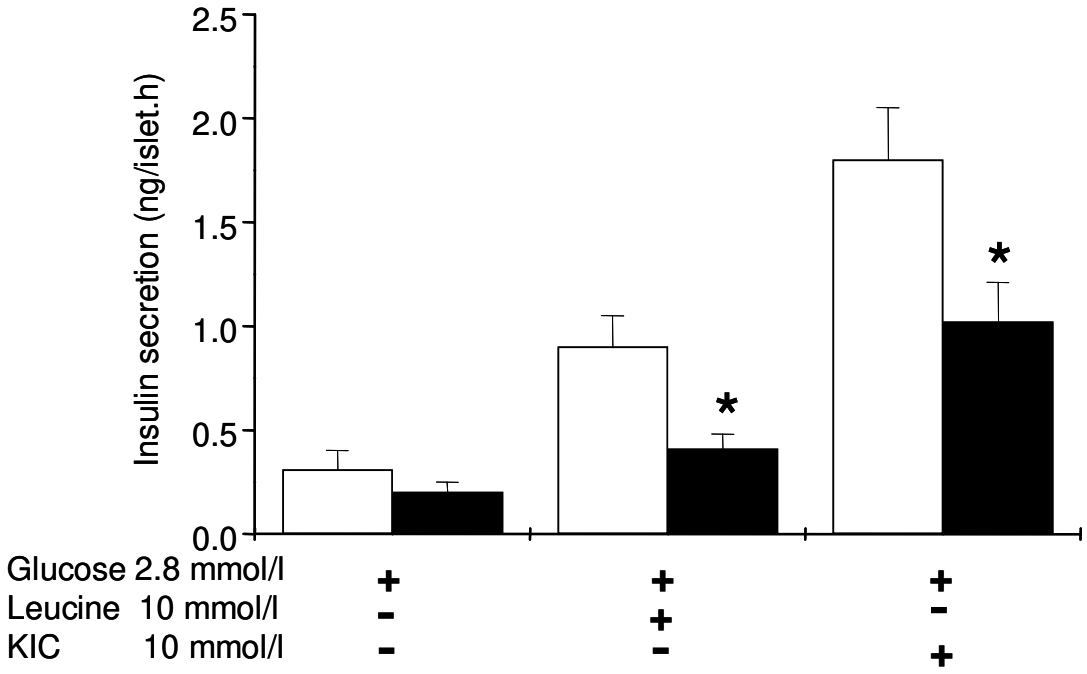

2.8 mmol/l
11.1 mmol/l
Insulin secretion (ng/50 islets)
10
8
3
2
1
0
20
30
40
50
60
70
80
90
100
Perfusion time (min)

Figure 4


Insulin secretion (ng/islet.h)
2.5
2.0
1.5
1.0
0.5
0.0
*
*
Glucose 2.8 mmol/l
Leucine 10 mmol/l
KIC 10 mmol/l
+ - -
+ + -
+ - +

VI.2 – Artigo 2 (Manuscrito): *"Lower expression of PKAα impairs insulin secretion in isolated islets from low-density lipoprotein receptor knockout mice"*

Lower expression of PKA∝ impairs insulin secretion in islets isolated from low-density lipoprotein receptor knockout mice

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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 investigated the possible mechanisms involved in such a defect in isolated $LDLR^{-/-}$ mice islets. 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 islets, suggesting a defect downstream of the substrate metabolism step of the insulin secretion pathway. After 5 min incubation, at 11.1 mmol/l glucose, ^{45}Ca uptake was higher in $LDLR^{-/-}$ than control islets. No differences in ^{45}Ca uptake was observed after 1 h incubation between groups. Insulin secretion induced by the PKA activators, forskolin and IBMX, in the presence of 11.1 mmol/l glucose, was lower in $LDLR^{-/-}$ islets, and was normalized in the presence of the PKC pathway activators, carbachol and PMA. Western blotting analysis showed that phospholipase C-β2 expression was increased and PKA-α decreased in $LDLR^{-/-}$ compared with control islets. In conclusion, these results indicate that the lower insulin secretion observed in islets from $LDLR^{-/-}$ mice at post-prandial concentrations of glucose can be explained, at least in part, by reduced expression of PKAα in these islets.

Key words: insulin secretion; isolates pancreatic islets; LDL receptor knockout mice

Introduction

The incidence of type 2 diabetes (T2DM) has reached international epidemic dimensions and is expected to affect 300 million people within the next 25 years (Fujimoto 2000). Numerous prospective cohort studies have indicated that T2DM is associated with a three-to fourfold increase in the risk for coronary heart disease (CHD) (Haffner 1998; Wilson 1998). Patients with T2DM have a 50% greater hospital mortality, and a two-fold higher rate of death within 2 years post myocardial infarction. Overall, CHD is the leading cause of death in individuals with T2DM (Ginsberg, Zhang et al. 2005).

Much of this increased CHD is associated with the presence of well-characterized risk factors, including abnormalities of plasma lipid and lipoprotein concentrations. The diabetic dyslipidemia is characterized by elevated blood levels of triglycerides (TG), low levels of high density lipoprotein (HDL) and increased or normal levels of low density lipoproteins (LDL) (Ginsberg 1991). Chemically altered LDL (oxidized and glycated), as well as smaller and denser LDL particles, are also features of the diabetic dyslipidemia (Cohen, Jin et al. 2004; Caparevic, Kostic et al. 2006).

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 the susceptibility to T2DM. Hypercholesterolemic LDL receptor knockout mice ($LDLR^{-/-}$), a model of human familial hypercholesterolemia (Ishibashi, Brown et al. 1993; Breslow 1996), when submitted to a Western-type diet, develop hypertriglyceridemia and obesity,

hyperglycemia and insulin resistance (Merat, Casanada et al. 1999), and severe atherosclerosis (Schreyer, Vick et al. 2002).

We have 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 post prandial glucose concentrations. The islets from these mice presented reduced glucose-derived CO_2 production, probably due to the increased substrate demand to sustain increased steroidogenesis (Bonfleur, Vanzela et al. 2007). Here, we investigate additional possible mechanisms involved in the lower insulin secretion observed in isolated LDLR$^{-/-}$ mice islets.

Insulin secretion from pancreatic β-cells is regulated by various factors, including fuel metabolism, hormones and neurotransmitters (Ahren, Sauerberg et al. 1999; Amaral, Ueno et al. 2003). Several modulators of insulin release act by activating protein kinases (PK) and phosphatases (Nesher, Anteby et al. 2002). The contribution of PKA to insulin secretion has been suggested to be relevant to the etiology of diabetes mellitus (Thorens and Waeber 1993; Ashcroft, Proks et al. 1994; Dachicourt, Serradas et al. 1996). PKA is a heterotetramer composed of two regulatory (R) and two catalytic (C) subunits. Increased availability of cAMP results in its binding to the R subunits, causing the release of the active C subunits that phosphorylate specific substrate proteins (Tasken and Aandahl 2004). PKA catalyzes the phosphorylation of many proteins in β-cells (Sugden, Ashcroft et al. 1979), including some required for insulin secretion such as those involved in the exocytotic machinery (Takahashi, Kadowaki et al. 1997).

Phospholipase C is present in rat and mouse pancreatic β-cells (Zawalich, Bonnet-Eymard et al. 2000) and specifically hydrolyzes plasma membrane phosphoinositides, forming

diacylglycerol (DAG) and inositide triphosphate (IP3) (Malaisse 1986). It is well known that DAG stimulates PKC activity and IP3 releases Ca^{2+} from internal stores, and both are important elements for insulin secretion.

In this study we investigated the possible involvement of the PKA and PKC pathways in the reduced insulin secretion response to glucose found in hypercholesterolemic $LDLR^{-/-}$ mice islets.

Methods

Animals - LDLR$^{-/-}$ mice 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 Universidade Estadual de Campinas. Animal experiments were approved by the university's Committee for Ethics in Animal Experimentation (CEEA/UNICAMP). The mice had free access to standard laboratory rodent chow diet (Nuvital CR1, Colombo, Paraná, Brazil) and water *ad libitum* and were housed at 22 ± 1ºC on a 12 h light/dark cycle. Both male and female mice of 16 - 20 weeks of age were used for the experiments.

Pancreatic islet isolation and static insulin secretion - 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. After isolation, batches of 4 islets each were pre-incubated in 1 ml of Krebs-Ringer bicarbonate buffer (KRBB) containing, in mmol/l: 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% BSA for 30 min at 37ºC. The islets were further incubated for 1 h in KRBB containing different secretagogues, as indicated in the results section. Aliquots of the supernatant (media) at the end of the incubation period were kept at –20ºC for posterior insulin measurement by radioimmunoassay.

Uptake of ^{45}Ca by isolated islets - Groups of 150 to 200 islets, derived from the same batch of islets, were preincubated for 30 min at 37°C in a medium containing 2.8 mmol/l glucose, pH 7.4. The incubation medium contained (in mmol/l): 115 NaCl; 5 KCl; 1 $CaCl_2$ and 1 $MgCl_2$, buffered with 10 mmol/l HEPES and bubbled with pure O_2. The islets were then incubated for

5 and 60 min in 200 µL of the same medium containing $^{45}CaCl_2$ (60 µCi/m
mmol/l glucose. At the end of the incubation period, 800 µL of ice-cold medium containing 2 mmol/l $LaCl_3$ (pH 7.4) was added to stop the reaction. The medium was then removed and an aliquot was saved to determine the amount of ^{45}Ca in the solution. The islets were subsequently washed three times with fresh, ice-cold La^{3+}-containing medium. The islets were then placed in a Petri dish and transferred (groups of ten islets) to counting vials containing 1 mL of ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA, 0.5 mmol/l). The uptake of ^{45}Ca was expressed as pmol Ca^{2+} per islet per min.

Western blotting After isolation by collagenase digestion of pancreata and subsequent separation on a discontinuous Ficoll DL-400 gradient, groups of islets were precipitated by centrifugation (750 x g for 10 min) and then resuspended in 50-100 µL of homogenization buffer (in mmol/l): 100 Tris, 10 sodium pyrophosphate, 100 sodium fluoride, 10 EDTA, 10 sodium vanadate, and 2 PMSF, and 1% SDS, pH 7.4. The islets were sonicated for 30 s using a Polytron PT 1200 C homogenizer (Brinkmann Instruments, NY) and the protein concentration was assayed using the Bradford method with BioRad reagent. The disrupted cells were treated with Laemmli sample buffer containing dithiothreitol and boiled for 5 min. Seventy µg of protein from each sample were loaded in a 10% polyacrylamide gel containing SDS and electrophoresed in a BioRad miniature slab gel apparatus. The electrotransfer of proteins from the gel to nitrocellulose was performed at 120 V for 1 h in a BioRad miniature transfer apparatus. The nitrocellulose filters were treated with a blocking buffer (5% non-fat dried milk, 10 mmol/l Trizma, 150 mmol/l NaCl, and 0.02% Tween 20) for 2 h at 22°C. The membranes were incubated for 4 h at 22°C with primary antibodies against phospholipase C

β2 (1:500), PKAα (1:1000), and actin (1:1000) (Santa Cruz, CA, USA), di

buffer with 3% non-fat dried milk and then washed for 30 min in buffer without milk. The blots were subsequently incubated with peroxidase-conjugated secondary antibody (Invitrogen, CA, USA) for 1 h in 2% non fat milk buffer and washed 3 times with buffer without milk. Specific protein bands were detected using 1 mL enhanced chemiluminescence reagents (Pierce Biotechnology, USA) and exposure to photographic film (Kodak, AM, Brazil). The intensities of the bands were quantified by optical densitometry (Scion Image, Frederick, MD).

Statistical analysis Results are presented as means ± SEM for the number of islet batches. The data were analyzed by unpaired Student's t test and the level of significance was set at $P < 0.05$.

Results

Animal features

Low fat chow fed female and male mice of 20 weeks of age, $LDLR^{-/-}$ and wild type (WT) mice were used in this study. As previously reported (Bonfleur, Vanzela et al. 2007), $LDLR^{-/-}$ mice present increased levels of total cholesterol (167%) and triglycerides (80%) when compared with WT mice, whereas free fatty acids levels are similar to those of the control group.

Insulin secretion induced by depolarizing agents

In the presence of sub-threshold (2.8 mmol/l) (Figure 1A) and post-prandial glucose concentrations (11.1 mmol/l) (Figure 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 ($P < 0.05$). At 2.8 mmol/l glucose, the insulin secretion was not different between groups (0.12 ± 0.01 and 0.11 ± 0.01 ng/islet.h 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/islet.h, respectively; n = 20-22; $P<0.05$).

Glucose-induced ^{45}Ca uptake

In order to analyze possible alterations in the Ca^{2+} handling by the $LDLR^{-/-}$ islets, we measured ^{45}Ca uptake during 5 min and 1 h incubation periods. After 5 min and at 11.1 mmol/l glucose, the ^{45}Ca uptake was higher in $LDLR^{-/-}$ than in WT islets (1.16 ± 0.05 and 0.75

± 0.05 pmol/islet.5 min, respectively; n = 29-35; $P < 0.05$). After a 60 min incubation, the ^{45}Ca uptake was similar in both groups (2.00 ± 0.2 and 1.85 ± 0.15 pmol/islet.h in $LDLR^{-/-}$ and WT islets; n = 17-25).

Carbachol (Cch) and phorbol 12-myristate 13-acetate (PMA) induced insulin secretion

When the islets were exposed to increasing concentrations of Cch (1 – 100 μmol/l), which stimulates the formation of IP3 and DAG through activation of phopholipase C, in the presence of 11.1 mmol/l glucose, the insulin secretion was dose-dependently similarly increased in both groups of islets. The levels of secretion observed at all concentrations of Cch in $LDLR^{-/-}$ were similar to those of WT islets (Figure 2 A). In addition, the presence of 100 nmol/l PMA, which stimulates PKC, also fully recovered insulin secretion in $LDLR^{-/-}$ islets (Figure 2 B).

Forskolin and 3-isobutyl-1-methyl-xanthine (IBMX) induced secretion

Elevation of cAMP by forskolin (10 μmol/l), which stimulates adenylate cyclase, and by IBMX (1 mmol/l), an inhibitor of phosphodiesterases, significantly stimulated secretion in both groups of islets. However, the insulin secretion stimulated by forskolin was markedly lower in $LDLR^{-/-}$ than in WT islets (7.19 ± 0.63 and 15.01 ± 1.04 ng/islet.h, respectively; n = 15; $P<0.05$). Insulin secretion was also lower in $LDLR^{-/-}$ than in WT islets when stimulated by IBMX (14.5 ± 1.2 and 19.8 ± 1.7 ng/islet.h in $LDLR^{-/-}$ and WT islets, respectively; n=15; $P<0.05$) (Figure 3). These results clearly show that the cAMP pathway is involved in the lower secretory response of $LDLR^{-/-}$ islets to glucose.

Phospholipase (PL) C-β2 and PKA-α protein expression

Western blotting analysis (Figure 4) shows that the expression of PLCβ2 in $LDLR^{-/-}$ islets was 55% higher (n = 4; $P < 0.05$), whereas the expression of PKAα was 22% lower (n = 5; $P < 0.05$), when compared with WT islets.

Discussion

In addition to hypercholesterolemia, LDLR$^{-/-}$ mice submitted to diabetogenic diets demonstrate increased adiposity, hypertriglyceridemia and hyperglycemia and peripheral insulin resistance, indicating that the dyslipidemia induced by the LDLR mutation and unbalanced diets impair glucose homeostasis (Merat, Casanada et al. 1999; Schreyer, Vick et al. 2002). 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 post-prandial hyperglycemia and hypoinsulinemia and glucose intolerance. Furthermore, we demonstrated that the main cause for the impaired glucose homeostasis was a reduced islet insulin secretion in response to glucose. The islets from these mice showed reduced glucose derived CO_2 production, probably due to the increased demand for substrate to sustain a higher steroidogenesis rate. Here, we investigated possible additional mechanisms involved in the lower secretory response of LDLR$^{-/-}$ mice islets.

First, we observed that depolarizing the β-cell membrane with high concentrations of KCl, $_L$-arginine and tolbutamide resulted in lower insulin secretion by LDLR$^{-/-}$ than by WT mice islets, both at basal and stimulatory glucose concentrations. The lower secretion observed in LDLR$^{-/-}$ islets was not due to islet insulin and DNA content (Bonfleur, Vanzela et al. 2007), but rather, may be a consequence of other defects in the signal transduction cascades leading to the secretory process. Glucose stimulation of insulin secretion occurs by a synergistic interaction between at least two signaling pathways. One, the 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, increasing $[Ca^{2+}]_i$ and, ultimately, activating the extrusion of insulin granules. In the second pathway, the ATP-sensitive K^+

channel-independent pathway, glucose still increases insulin secretion without affecting the membrane potential (also known as the Ca^{2+}-dependent pathway). Since glucose metabolism was reduced in $LDLR^{-/-}$ islets by 30% (Bonfleur, Vanzela et al. 2007), the ATP/ADP ratio in these islets may be reduced, leading to a lower degree of cell membrane depolarization and, consequently, a lower Ca^{2+} uptake. The amount of ^{45}Ca taken up by these islets during the first 5 min was actually higher, however, after 1 hour incubation, it was not different from that observed in WT islets. Thus, the lower insulin secretion in $LDLR^{-/-}$ islets, which was not corrected by additional membrane depolarization, may be due to alterations in the ATP-sensitive K^+ channel-independent pathway (Gembal, Gilon et al. 1992; Sato, Aizawa et al. 1992; Gembal, Detimary et al. 1993).

Activation of PKC is important for the phosphorylation of many proteins associated with the exocytotic process in β-cells (Jones and Persaud 1998). 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 (Boschero, Szpak-Glasman et al. 1995). Here, we observed that Cch restored the insulin secretory competence in $LDLR^{-/-}$ islets, reaching a degree even higher than that of WT islets. This effect is in close agreement with a higher PLC-β2 expression found in $LDLR^{-/-}$ islets. Although the expression of PKCα was not altered in $LDLR^{-/-}$ islets (not shown), we also observed a fully recovered 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, non-stimulatory glucose concentrations; however this is not enough to cope with the full response to post prandial levels of glucose. The increased insulin

secretion induced by Cch is, at least in part, dependent on the augmented cytoplasm Ca^{2+} concentrations induced by the agent.

The importance of the cAMP/PKA pathway for the process of insulin secretion has long been demonstrated (Malaisse, Pipeleers et al. 1974). Several hormones (incretins) utilize this pathway to potentiate glucose-stimulated insulin secretion (Szecowka, Grill et al. 1982; Thorens 1994; Huypens, Ling et al. 2000). This potentiation involves a series of reactions triggered by PKA, including the phosphorylation of vesicular and plasma membrane proteins, voltage-dependent channels, and transcription factors (Jones and Persaud 1998). PKA activity seems to be specifically involved in the granule fusion reaction (Hatakeyama, Kishimoto et al. 2006), since reduction of PKA expression diminishes the phosphorylation of proteins involved in the extrusion of insulin granules, such as snapin (Chheda, Ashery et al. 2001) and SNAP25 (Nagy, Reim et al. 2004). Supporting this view, reduced glucose-induced insulin secretion by isolated islets and insulinoma cells is observed by inhibition of PKA (Wang, Zhou et al. 2001) and lower PKA-α expression (Ferreira, Barbosa et al. 2004; Milanski, Arantes et al. 2005). Thus, the concomitant reduction of PKA-α expression and insulin secretion observed in $LDLR^{-/-}$ mice islets 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 (GIE) (Hatakeyama, Kishimoto et al. 2006). PKA inhibitors did not affect the glucose-induced increase in $[Ca^{2+}]_i$ and the exposure of islets to high glucose concentrations increased the duration of Ca^{2+}-dependent insulin exocytosis (CIE), an effect not mimicked by 2-deoxy-D-glucose, indicating that a glucose metabolite is necessary for the process (Hatakeyama, Kishimoto et al. 2006). One candidate is ATP (Takahashi, Kadowaki et al. 1999) given that glucose rapidly increases

its cytosolic concentrations (Henquin 1990). The fast mode of CIE is augmented by intracellular levels of ATP in a concentration-dependent manner (Kasai, Suzuki et al. 2002) and this effect seems to require both cAMP and PKA. Activation of PKA was also observed by increasing intracellular levels of ATP even in the absence of increased concentrations of cAMP (Takahashi, Kadowaki et al. 1999). In our experiments, both forskolin and IMBX, which increase intracellular cAMP concentrations, failed to fully restore the insulin secretory competence in $LDLR^{-/-}$ islets, indicating a lower availability of PKA for activating the secretion process. As stated before, we have previously observed a 30% reduction in the glucose metabolism in $LDLR^{-/-}$ islets, suggesting a possible reduction in intracellular levels of ATP. Since ATP and PKA are important for the final steps of the 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, previous (Bonfleur, Vanzela et al. 2007) and present data indicate that the lower insulin secretion in $LDLR^{-/-}$ mice islets may be explained by reduced glucose metabolism and expression of PKA. The mechanism responsible for reduction in PKA expression in LDL receptor defective islets remains to be elucidated.

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Figure Legends

Figure 1. Insulin secretion induced by 40 mmol/l KCl, 10 mmol/l $_L$-arginine and 100 µmol/l tolbutamide in islets from WT (*open bars*) and LDLR$^{-/-}$ mice (*dark bars*) in the presence of 2.8 mmol/l glucose (1A) and 11.1 mmol/l glucose (1B). Groups of 4 islets of similar sizes were incubated for 1 h at 2.8 mmol/l or 11.1 mmol/l glucose plus depolarizing agents. Each bar represents mean ± SEM of 10-25 groups of islets.*$P < 0.05$ related to respective control.

Figure 2. Insulin secretion induced by carbachol (1-100 µmol/l) and 100 nmol/l PMA in islets from WT (*open bars*) and LDLR$^{-/-}$ mice(*dark bars*). Groups of 4 islets of similar sizes were incubated for 1 h at 11.1 mmol/l glucose. Each bar represents mean ± SEM of 15 groups of islets.*$P < 0.05$ related to respective control at the same concentrations of carbachol and PMA.

Figure 3. Insulin secretion induced by 10 µmol/l forskolin and 1 mmol/l IBMX in islets from WT (*open bars*) and LDLR$^{-/-}$ mice (*dark bars*). Groups of 4 islets of similar sizes were incubated for 1 h at 11.1 mmol/l glucose. Each bar represents mean ± SEM of 14-15 groups of islets.*$P < 0.05$ related to respective control.

Figure 4. PLCβ2 and PKAα expression in islets from WT (*open bars*) and LDLR$^{-/-}$ mice (*dark bars*). Protein extracts were processed for Western blot detection of PLCβ2 (**A**) and PKAα **(B)**. The bars represent the means ± SEM of the values, determined by optical densitometry. n=4-5; *$P < 0.05$ *vs*. WT.

Figure 1

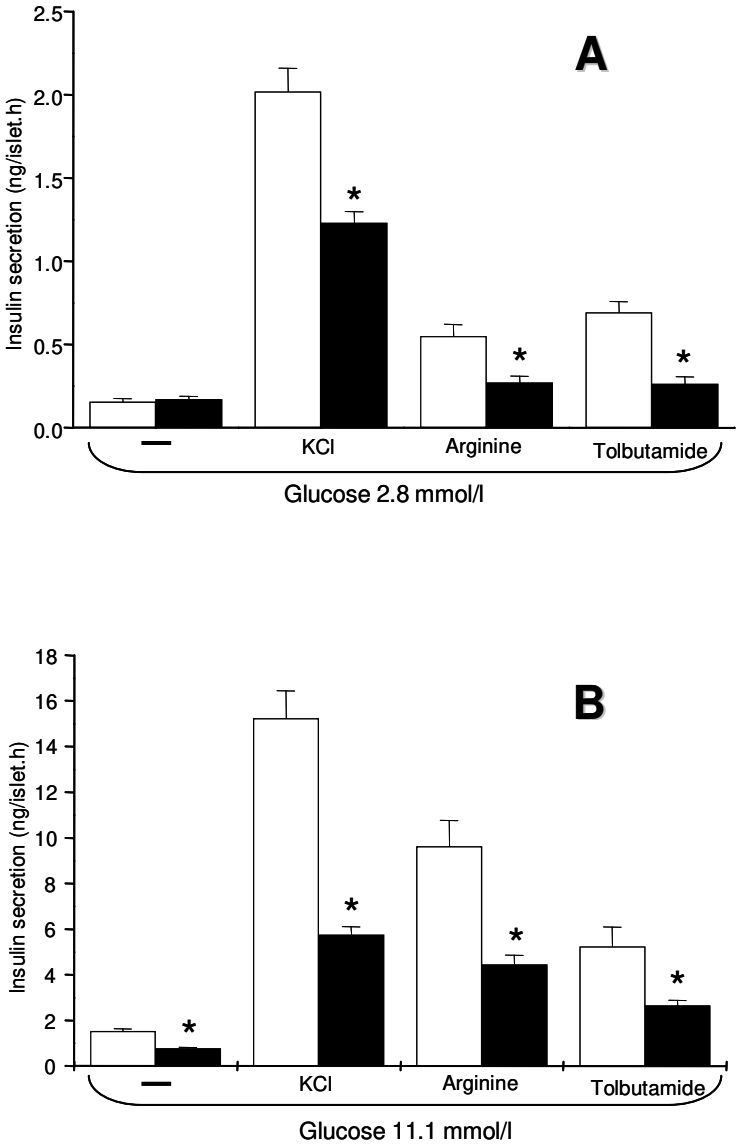
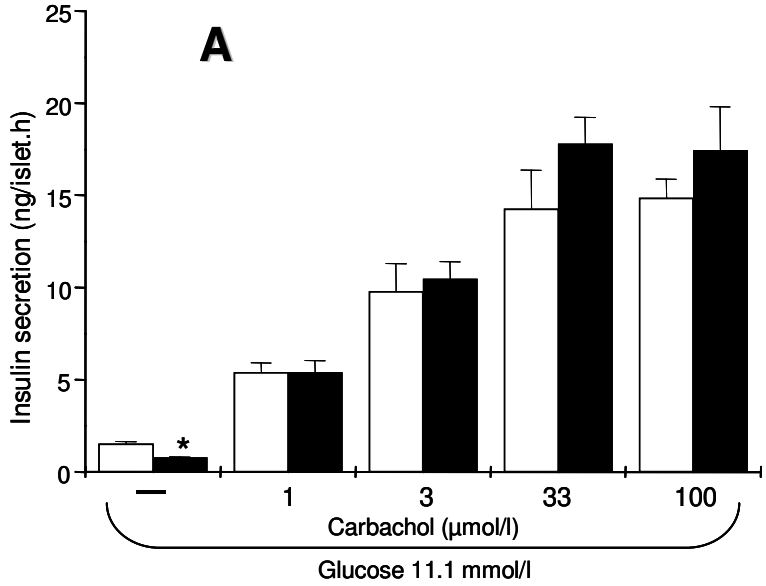

A
Insulin secretion (ng/islet.h)
2.5
2.0
1.5
1.0
0.5
0.0
*
*
*
—
KCl
Arginine
Tolbutamide
Glucose 2.8 mmol/l
B
Insulin secretion (ng/islet.h)
18
16
14
12
10
8
6
4
2
0
*
*
*
*
—
KCl
Arginine
Tolbutamide
Glucose 11.1 mmol/l

Figure 2


A
Insulin secretion (ng/islet.h)
25
20
15
10
5
0
*
—
1
3
33
100
Carbachol (µmol/l)
Glucose 11.1 mmol/l

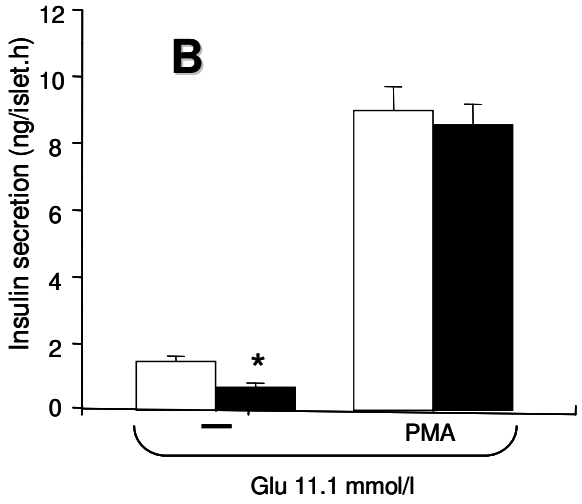

B
Insulin secretion (ng/islet.h)
12
10
8
6
4
2
0
*
—
PMA
Glu 11.1 mmol/l

Figure 3

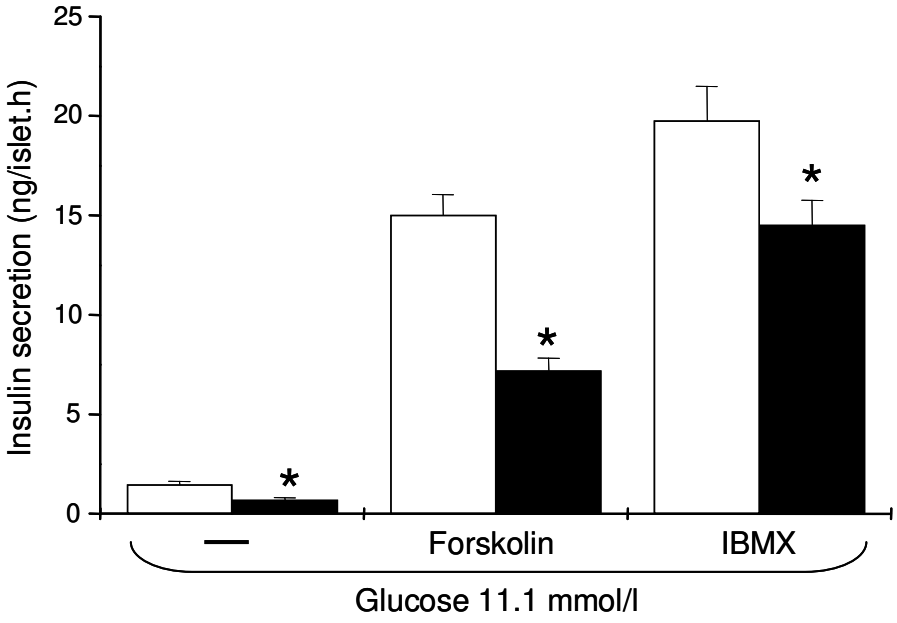
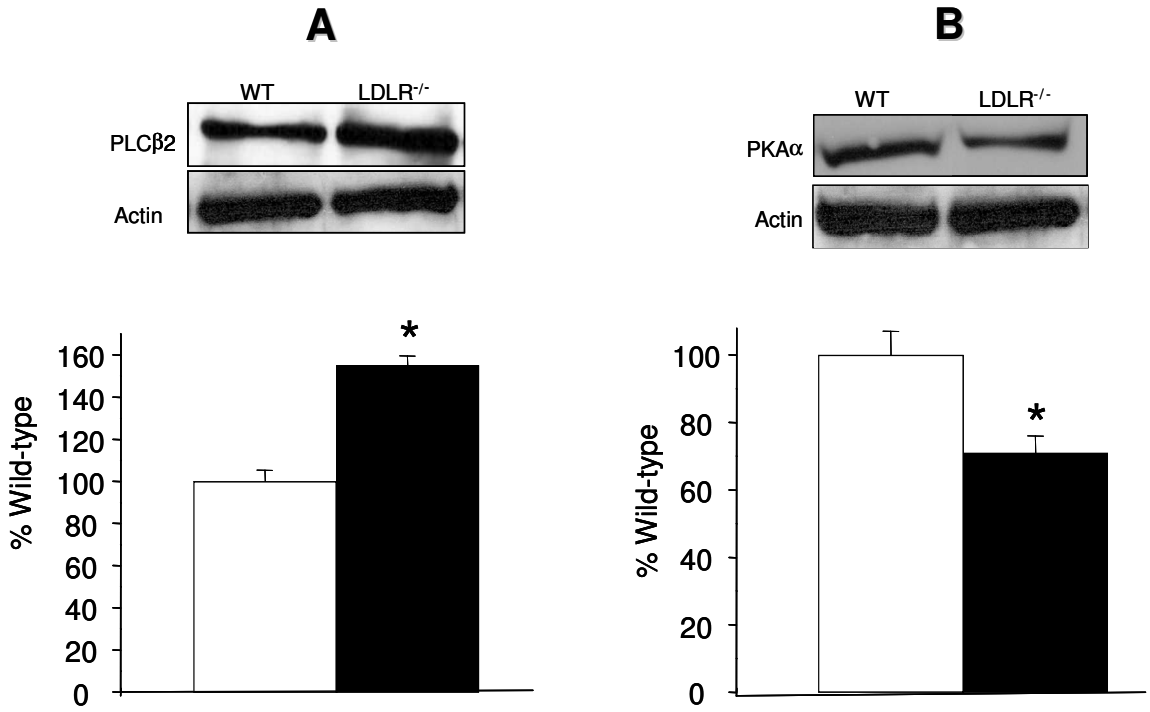

25
20
15
10
5
0
Insulin secretion (ng/islet.h)
*
*
*
—
Forskolin
IBMX
Glucose 11.1 mmol/l

Figure 4


A
WT
LDLR-/-
PLCβ2
Actin
% Wild-type
160
140
120
100
80
60
40
20
0
*
B
WT
LDLR-/-
PKAα
Actin
% Wild-type
100
80
60
40
20
0
*

VI – CONCLUSÕES

Observamos que camundongos *knockout* para o receptor de LDL apresentam alterações na homeostase glicêmica independente de dieta rica em gordura e são intolerantes à glicose. No entanto, a resistência periférica global à ação da insulina está normal nos animais *knockouts* tanto jovens como envelhecidos. A intolerância à glicose é decorrente de uma redução na capacidade de secreção de insulina pelas ilhotas pancreáticas, visto que as ilhotas destes animais apresentaram redução na liberação deste hormônio na presença de glicose, bem como, de outros secretagogos metabolizáveis, agentes despolarizantes e ativadores da PKA. As ilhotas destes camundongos também apresentam uma redução na oxidação da glicose e aumento do metabolismo da leucina, provavelmente, decorrente de um desvio destes substratos para a via da esteroidogênese, a qual se encontra aumentada nestes animais. Ainda, a secreção de insulina está prejudicada nas ilhotas dos camundongos *knockout* por redução da expressão protéica da proteína-quinase dependente de AMP cíclico (PKA).

Assim, concluímos que a deficiência de receptores de LDL *per se* leva à alteração da homeostase glicêmica por redução na secreção de insulina. Esta é decorrente de pelo menos dois mecanismos: 1- diminuição da metabolização da glicose no ciclo de Krebs, o que provavelmente resulta em menor produção de ATP, e 2- redução da expressão da PKA, comprometendo esta via de sinalização para secreção de insulina nas ilhotas destes animais.

VIII – ANEXOS

Aprovação do Comitê de Ética em Experimentação Animal e da Comissão Interna de Biossegurança

DECLARAÇÃO

Declaro para os devidos fins que o conteúdo de minha dissertação/ tese de mestrado/doutorado intitulada "**Efeito da hipercolesterolemia genética sobre a homeostase glicêmica e secreção de insulina em camundongos *knockout* para o receptor de LDL ($LDLR^{-/-}$)**"

( ) não se enquadra no Artigo 1°, § 3° da Informação CCPG 002/06, referente a bioética e biossegurança.

(X) está inserido no Projeto CIBio (Protocolo n° 2001/07), intitulado "Metabolismo de lipoproteínas ricas em triglicérides em camundongos transgênicos"

(X) tem autorização da Comissão de Ética em Experimentação Animal (Protocolo n° 1108-1) aprov 25/10/06

( ) tem autorização do Comitê de Ética para Pesquisa com Seres Humanos.

Aluno Maria Lúcia Bonfleur

Orientador
Helena Coutinho Franco de Oliveira

Para uso da Comissão ou Comitê pertinente:

(X) Deferido ( ) Indeferido

Nome:
Função: Profa. Dra. ANA MARIA A. GUARALDO
Presidente
Comissão de Ètica na Experimentação Animal
CEEA/IB - UNICAMP

Profa. Dra. HELENA COUTINHO F. DE OLIVEIRA
Presidente
Comissão Interna de Biossegurança
CIBio/IB - UNICAMP

MARIA SILVIA VICCARI GATTI
MEMBRO TITULAR CIBio/IB/UNICAMP
20/08/2007

UNICAMP

Universidade Estadual de Campinas
Instituto de Biologia

CEEA-IB-UNICAMP

Comissão de Ética na Experimentação Animal
CEEA-IB-UNICAMP

CERTIFICADO

Certificamos que o Protocolo nº **1108-1**, sobre **"EFEITO DE HIPERLIPIDEMIAS GENÉTICAS SOBRE A HOMEOSTASE GLICÊMICA: ESTUDOS EM CAMUNDONGOS GENETICAMENTE MODIFICADOS"**, sob a responsabilidade de **Profa. Dra. Helena C. F. Oliveira / Maria Lúcia Bonfleur**, está de acordo com os Princípios Éticos na Experimentação Animal adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA), tendo sido aprovado pela Comissão de Ética na Experimentação Animal (CEEA)-IB-UNICAMP em reunião de 25 de outubro de 2006.

CERTIFICATE

We certify that the protocol nº **1108-1**, entitled **"EFFECTS OF PRIMARY HYPERLIPIDEMIAS ON THE GLYCEMIC HOMEOSTASIS: STUDIES IN GENETICALLY MODIFIED MICE"**, is in agreement with the Ethical Principles for Animal Research established by the Brazilian College for Animal Experimentation (COBEA). This project was approved by the institutional Committee for Ethics in Animal Research (State University of Campinas - UNICAMP) on October 25, 2006.

Campinas, 25 de outubro de 2006.

Profa. Dra. Ana Maria A. Guaraldo
Presidente

Fátima Alonso
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