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"AVALIAÇÃO DOS EFEITOS DE DIFERENTES CONCENTRAÇÕES DE DEXAMETASONA SOBRE PARÂMETROS FISIOLÓGICOS DE ILHOTAS PANCREÁTICAS"

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

Orientador: Prof. Dr. José Roberto Bosqueiro

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DEDICATÓRIA

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Bernardo Houssay

"Não deixe que a saudade sufoque, que a rotina acomode, que o medo impeça de tentar. Desconfie do destino e acredite em você. Gaste mais horas realizando que sonhando, fazendo que planejando, vivendo que esperando porque, embora quem quase morre esteja vivo, quem quase vive já morreu."

Luiz Fernando Veríssimo

A resistência à insulina (RI) é uma condição que exige maiores níveis de insulina circulante e que normalmente são providenciados pelo aumento da função e população de células β. A RI pode ser observada a partir de diversos modelos experimentais em roedores tais como os modelos transgênicos, de gravidez, submetidos às dietas hiperlipídicas e hipercalóricas e a partir de infusão venosa de glicose. Estes modelos têm sido úteis para a compreensão dos mecanismos compensatórios observados durante a RI. Os glicocorticóides são amplamente utilizados na indução farmacológica da RI em modelos animais e em seres humanos, com fins científicos. A ativação da sinalização da insulina e das proteínas reguladoras do ciclo celular é crucial para a função e crescimento das células β adultas. No presente trabalho, apresentamos modelos para investigação da função e crescimento de células β pancreáticas *in vivo* a partir da administração diária de três concentrações distintas de dexametasona (DEX) (0,1, 0,5 e 1,0 mg/kg, peso corpóreo, intraperitoneal - DEX 0.1, DEX 0.5 e DEX 1.0, respectivamente) por 5 dias consecutivos. A sensibilidade periférica à glicose e à insulina, parâmetros de secreção de insulina e histomorfométricos foram investigados. A análise dos níveis de proteínas relacionados à função e crescimento de células β foi realizada por Western blotting. O tratamento com DEX induziu RI de maneira dose-dependente. Aumento da secreção de insulina em resposta à glicose foi observado tanto *in vivo* quanto *ex vivo* nos três grupos tratados com DEX. Ratos DEX 1.0, que apresentam hiperglicemia moderada e marcante hiperinsulinemia, exibiram aumento de 5,1 vezes na proliferação além de hipertrofia de células β, com aumento significativo na massa de células β comparado aos ratos CTL. Os ratos DEX 0.5, hiperinsulinêmicos, porém normoglicêmicos, também apresentaram aumento significante de 3,6 vezes na proliferação e modesta hipertrofia de células β. Entretanto, os ratos DEX 0.1, que desenvolveram o menor grau de RI, compensaram à demanda de insulina apenas com

aumento da função de células β. Nenhuma alteração da freqüência de morte celular foi observada nas células β dos três grupos DEX comparados ao grupo CTL. Foi observada ativação da via IRS-2/PI3-K/Akt/$p70^{S6K}$, bem como da proteína retinoblastoma nas ilhotas do grupo DEX 1.0 e, em menor grau, no grupo DEX 0.5 quando comparados com as ilhotas do grupo CTL. Assim, aumentando a concentração de dexametasona induzem-se três graus de requerimento de insulina *in vivo*, servindo como modelo para investigação de alterações compensatórias em células β. O aumento da demanda de insulina é compensado por aumento da função das células β (em todos os GRUPOS DEX) e por hiperplasia e hipertrofia de células β nos GRUPOS DEX 1.0 e DEX 0.5. Baseado nos presentes resultados concluímos que o aumento dos níveis circulantes de insulina parece ser o maior estímulo para proliferação e hipertrofia das células de células β observado na RI induzida pela dexametasona.

Insulin resistance (IR) is a condition that demand increased levels of circulating insulin that are normally provided by increase of β-cell function and mass. The IR can be observed in several experimental rodent models such as transgenic, pregnancy, high-fat or high-caloric diet and from glucose infusion model. These models have aided in elucidating the compensatory mechanisms observed during the IR. The glucocorticoids are widely used to induce the pharmacological IR in animal models and in humans, with scientific purpose. Activation of insulin signaling and cell cycle proteins are crucial to the function and growth of adult β cells. At the present study, we showed models to investigation of pancreatic β-cell function and growth *in vivo* from the daily administration of three different dexamethasone (DEX) concentration (0.1, 0.5 e 1.0 mg/kg, body weight, intraperitoneal - DEX 0.1, DEX 0.5 and DEX 1.0, respectively) for 5 consecutive days. The peripheral sensibility to glucose and insulin, insulin secretion and histomorphometrical parameters were investigated. The analyses of proteins related to β-cell function and growth were done by Western blotting. DEX treatment induced IR in a dose-dependent manner. Incease of glucose-stimulated insulin secretion was observed *in vivo* as well as *ex vivo* in the three DEX groups. DEX 1.0 rats, that present moderate hyperglicemya and marked hyperinsulinemia, ehibited a 5.1-fold increase in β-cell proliferation besides hypertrophy, with significant increase of β-cell mass compared to CTL rats. DEX 0.5 rats, that are hiperinsulinemic and normoglicemic, also exhibited a significant 3.6-fold increase in β-cell proliferation as well as β-cell hypertophy. However, DEX 0.1 rats, which exhibited the lowest degree of insulin resistance, compensate for insulin demand by improving only β-cell function. No alteration in cell death frequency was noted in β-cells from the three DEX groups compared to CTL group. Activation of IRS-2/PI3-K/Akt/$p70^{S6K}$ pathway as well as the retinoblastoma protein in islets from DEX 1.0 and, in lesser extend, in DEX 0.5 group was observed compared to islets from CTL group. Therefore,

increasing doses of dexamethasone induce three different degrees of insulin requirement in living rats, serving as a model to investigate compensatory beta-cell alterations. The increased insulin demand is compensated by increase of β-cell function (in all DEX groups) and β-cell hyperplasia and hypertrophy in DEX 0.5 and DEX 1.0 groups. Based on the present results we concluded that the augmented levels of circulating insulin seem to be the major stimulus for β-cell proliferation and hypertrophy observed in dexamethasone-induced insulin resistance.

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LISTA DE ABREVIATURAS

AC *adenilil ciclase*

ADP *adenosina difosfato*

Akt *proteína cinase serina-treonina*

ATP *adenosina trifosfato*

Bcl-2 *proteína proto-oncogênica com nome derivado de linfoma de célula B-2*

cAMP *adenosina monofosfato cíclico*

Cdk4/6 *cinase dependente de ciclina 4/6*

CIP *proteína inibitória de ciclina*

DAG *diacilglicerol*

DMT1/2 *diabetes mellitus tipo 1/2*

E2F *fator de transcrição de elongamento 2*

ERK *cinase regulada por sinal extracelular*

$FADH_2$ *dinucleotídeo de flavina e adenina*

FKHR *fatores de transcrição tipo "forkhead"*

GAD65 *descarboxilase de glutamato 65*

GLUT2/4 *transportador de glicose 2/4*

Grb2 *proteína ligadora do receptor de fator de crescimento 2*

GSK-3 *cinase de proteína glicogênio sintase*

G-6-P *glicose 6 fosfato*

IA-2 *antígeno de insulinoma-2*

IGF-1 *fator de crescimento semelhante a insulina-1*

INK *cinase inibitória*

ipGTT *teste de tolerância à glicose intraperitoneal*

ipITT *teste de tolerância à insulina intraperitoneal*

IRS-1/2 *substrato do receptor de insulina ½*

IP_3 *trifosfato de inositol*

K_{ATP} *canal de potássio dependente de ATP*

KIP *proteína inibitória de cinase*

MAPK *proteína cinase ativada por mitógeno*

MAPKK *proteína cinase cinase ativada por mitógeno*

NADH *dinucleotídeo de nicotinamida e adenina*

nck *adaptador de citoesqueleto*

PCNA *antígeno nuclear de proliferação celular*

PDK-1 *proteína dependente de fosfatidilinositol*

PDX-1 *proteína "homeobox" duodenal-pancreática 1*

PI3-K *fosfatidilinositol 3-cinase*

PKA/C *cinase de proteína A/C*

PLC *fosfolipase C*

P70^{S6K} *proteína ribossomal cinase S6*

pRb *proteína retinoblastoma*

Ras/Raf *proteína ativada por GTPase*

RI *resistência periférica à insulina*

SH2/3 *domínio de homologia Src 2/3*

Src *família de proteínas cinase de tirosina proto-oncogênicas*

SHP2 *proteína fosfatase de tirosina*

SOS *"son-of-sevenless"*

Pâncreas Endócrino

Dois grandes sistemas são responsáveis pela monitoração e regulação das funções do corpo: o sistema nervoso e o sistema endócrino. Cada sistema é crucial para o funcionamento fisiológico cooperativo da multiplicidade de células altamente diferenciadas dos tecidos e órgãos que compõem o organismo humano. Ambos os sistemas, juntos, integram-se para que o organismo responda adequadamente às alterações de seu ambiente externo e interno. Em geral, o sistema endócrino está envolvido principalmente com as diferentes funções metabólicas do corpo, como a velocidade das reações químicas nas células e o transporte de substâncias através das membranas celulares bem como com outros aspectos do metabolismo celular, como crescimento e secreção. Para que estas funções metabólicas encontrem-se em harmonia, um conjunto de glândulas e seus respectivos produtos secretores (hormônios) são necessários. Dentre estes órgãos, o pâncreas, além de suas funções digestivas, sintetiza e secreta importantes hormônios, entre eles a insulina e o glucagon.

Estes hormônios são sintetizados e estocados em um microórgão especializado, as ilhotas pancreáticas de Langerhans (Ashcroft & Ashcroft, 1992). A primeira descrição deste microórgão ocorreu entre 1867 e 1868 através de estudos com pâncreas de coelhos desenvolvido ao longo do doutorado do pesquisador alemão Paul Langerhans. Com suas próprias palavras o pesquisador decreveu as células β pancreáticas como: "células pequenas, de conteúdo praticamente homogêneo e com forma poligonal, com núcleo arredondado e sem nucléolo e unidas sempre de duas em duas formando pequenos grupos" (Gimenez & Benito, 2003). Compreendendo aproximadamente 2% da massa total do pâncreas, em humanos, cada ilhota é composta por milhares de células, as células β – que sintetizam e secretam o hormônio insulina, as células α - que sintetizam e secretam o hormônio glucagon, as células γ - que sintetizam e secretam o hormônio somatostatina e as células PP – que sintetizam e

secretam o polipeptídeo pancreático (Kanno *et al.*., 2002; Porksen *et al.*, 2002). A insulina participa de inumeros processos metabólicos, entre os quais no controle do metabolismo dos carboidratos, das gorduras e das proteínas, desempenhando portanto, papel importante no armazenamento do excesso de substâncias energéticas. O caminho para a descoberta desse hormônio passou pelos trabalhos do fisiologista e bioquímico romano Nicolae Paulesco, que, chegou a elaborar um extrato pancreático que denominou de pancreatina em 1921. Mas foi com os trabalhos liderado pelo médico e pesquisador canadense Frederick Grant Banting, ajudado pelo professor escocês John Janes Richard Macleod e o médico norte-americano Charles Herbert Best que em julho de 1921 Banting conseguiu demonstrar os efeitos anti-diabéticos de seu extrato pancreático obtido do próprio pâncreas do cachorro que havia tornado diabético. A até então denominação de ilhotina atribuída por Banting para este composto anti-diabético foi trocada para insulina por sugestão de Macleod (Gimenez & Benito, 2003). A insulina está designada a monitorar o aumento nas concentrações plasmáticas de nutrientes, em especial o da glicose, no sangue. Em qualquer momento, em um organismo normal, a homeostasia da glicose é mantida pelo balanço entre a secreção e a ação da insulina, sendo a glicose sanguínea o principal substrato regulador deste processo secretório (Deeney *et al.*, 2000; Rutter, 2001).

Mecanismo Geral de Secreção de Insulina

A secreção de insulina é estimulada por substratos energéticos metabolizáveis pelas células β pancreáticas, sendo a glicose o secretagogo mais importante. A sinalização inicia-se com o transporte da glicose para o interior da célula β por uma proteína integral de membrana denominada transportador de glicose 2 (GLUT2). Na célula β a glicose é fosforilada e

convertida a glicose-6-fosfato (G-6-P) por duas enzimas: a hexoquinase I e a hexoquinase IV (glicoquinase). A primeira é fortemente inibida pela G-6-P e em menor grau pela frutose 1-6-difosfato. Assim, a glicoquinase, que não é inibível pela G-6-P, desempenha papel fundamental sobre a fosforilação de glicose nas células β. A enzima glicoquinase participa da regulação do fluxo glicolítico e, portanto, no processo de secreção de insulina, atuando como sensor de glicose nas células β (Boschero, 1996; Matchinky, 1996) (Figura 1). A G-6-P é preferencialmente destinada à glicólise e o substrato resultante deste processo, o piruvato, é transportado do citoplasma à mitocôndria, onde é convertido à acetil-CoA que segue para o ciclo de Krebs culminando na formação de nicotinamida adenina dinucleotídeo (NADH) e flavina adenina dinocleotídeo ($FADH_2$). Estas coenzimas servem como fonte para transferência de elétrons durante a fosforilação oxidativa mediando a produção de ATP e, com aumento da razão ATP/ADP intracelular (Ashcroft, 1980), ocorre o fechamento dos canais de potássio sensíveis ao ATP (K_{ATP}). Esse evento promove desequilíbrio iônico e resulta em despolarização da membrana da célula β com consequente ativação da permeabilidade aos íons Ca^{2+}. Estes íons penetram a célula por gradiente eletroquímico através de canais dependentes de voltagem específicos (canais Ca^{2+} tipo L). A elevação dos níveis intracelulares deste íon ativa a maquinaria secretória, ocorrendo migração dos grânulos de insulina para a membrana plasmática e posterior extrusão de seu conteúdo (Barg, 2002; Rorsman & Renström, 2003). Embora a glicose seja o principal substrato responsável pela estimulação da secreção de insulina, o mecanismo secretório pode ser modulado direta ou indiretamente, por hormônios, neurotransmissores e agentes farmacológicos (Figura 1). Esse controle multifatorial permite que as células β secretem insulina em quantidade e tempo adequados, regulando adequadamente os níveis de nutrientes no sangue em diferentes situações

fisiológicas, tais como: jejum, refeição, exercício, gravidez, lactação e crescimento (Boschero, 1996).

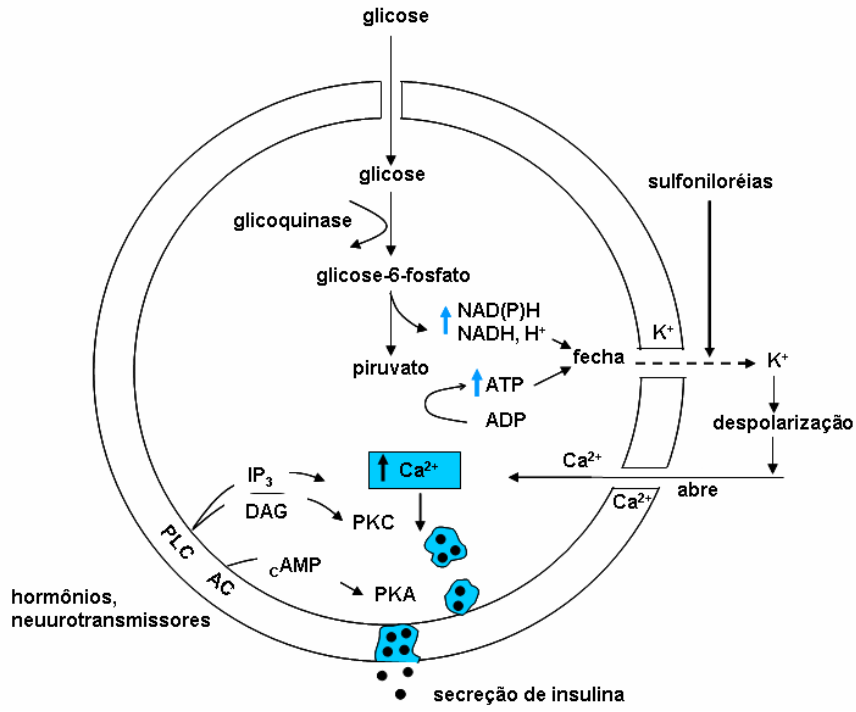

Figura 1 *Mecanismo de secreção de insulina* A entrada da glicose pela célula β induz o fechamento dos canais de K^+, despolarização e abertura de canais de Ca^{2+} dependentes de voltagem com conseqüente secreção de insulina. Outros fatores que modulam a secreção de insulina podem agir via estimulação de fosfolipase C (PLC) ou adenilil ciclase (AC) que ativa a via do inositol ou cAMP, repectivamente. DAG – diacilglicerol; IP_3 – inositol 1,4,5-trifosfato; PKA/C – protein cinase A/C.

Via de Sinalização da Insulina

Os efeitos fisiológicos da insulina iniciam-se após seu acoplamento com o receptor de membrana específico presente em praticamente todos os tecidos. O receptor de insulina é uma proteína com atividade cinase de tirosina, composta por duas subunidades α e duas subunidades β, unidas por pontes dissulfeto (Kahn, 1985) (Figura 2). A subunidade α se localiza na porção extracelular, possui o sítio para acoplamento da insulina e apresenta atividade inibitória sobre a subunidade β, inibindo sua atividade tirosina cinase. O

acoplamento da molécula de insulina com a subunidade α do receptor de insulina permite que a subunidade β adquira atividade tirosina cinase do receptor (Patti & Kahn, 1998).

A autofosforilação da subunidade β do receptor de insulina, induzida pelo acoplamento da insulina, desencadeia uma cascata de reações de fosforilações de substratos celulares, incluindo os membros da família de substratos do receptor de insulina (IRSs). As funções fisiológicas do substrato do receptor de insulina 1 (IRS-1) e do substrato do receptor de insulina 2 (IRS-2) foram demonstradas através de estudos derivados de camundongos transgênicos "knock out", ou seja, sem a presença sistêmica de cada um dos dois componentes. Camundongos deficientes em IRS-1 apresentam retardo no crescimento, mas não desenvolvem diabetes uma vez que a secreção de insulina aumenta para compensar a resistência à insulina (RI) moderada (Araki *et al.*, 1994; Tamemoto *et al.*, 1994). Por outro lado, camundongos deficientes em IRS-2 apresentam comprometimento tanto da sinalização da insulina em tecidos periféricos quanto da função das células β pancreáticas (Withers, 1998). Os camundongos IRS-$2^{-/-}$ apresentam deterioração progressiva da homeostase glicêmica por conta da RI presente no fígado e músculos esqueléticos bem como pela perda da compensação pelas células β (observada pela redução da massa de células β) frente a RI. A fosforilação em tirosina do IRS gera sítios de reconhecimento para moléculas contendo domínios com homologia a Src2 (SH2), incluindo proteínas como Grb2, nck, SHP2 e a fosfatidilinositol 3-cinase (PI3-K), entre outras (Burks & White, 2001). A associação da PI3-K com as proteínas IRSs é importante para o processo de translocação do transportador de glicose 4 (GLUT4), captação de glicose estimulada por insulina (Czech & Corvera, 1999), ativação da glicogênio sintetase, inibição da lipólise estimulada pela insulina (White, 1998) e ativação da expressão gênica e da síntese protéica (Kido *et al.*, 2001) (Figura 2). A via da proteína cinase da cinase ativada por mitógeno (MAPKK)/Ras pode ser ativada pela insulina

através da formação de complexos entre os fatores SOS e Grb2 que podem desempenhar papel em tecidos estimulando ações de crescimento e proliferação da insulina (Kido *et al.*, 2001). A PI3-K é constituída por duas subunidades, uma catalítica de 110 kDa e outra regulatória de 85 kDa. A subunidade regulatória possui dois domínios SH2 e um SH3 que interagem com os sítios de fosforilação dos IRSs (Myers *et al.*, 1992) dando início a uma série de eventos incluindo uma cascata de reações de fosforilações e desfosforilações que regula os efeitos metabólicos e de crescimento da insulina (Sun *et al.*, 1991; White & Kahn, 1994; White, 1997). Uma das proteínas alvo desta enzima é a proteína serina-treonina cinase Akt. Cho *et al.* (2001) e Downward (1998) demonstraram o papel importante da Akt na homeostase da glicose, crescimento e sobrevivência celular, além de fosforilação de proteínas que regulam a síntese de lipídeos, glicogênio e proteínas (Saltiel & Kahn, 2001; Kido *et al.*, 2001) (Figura 2).

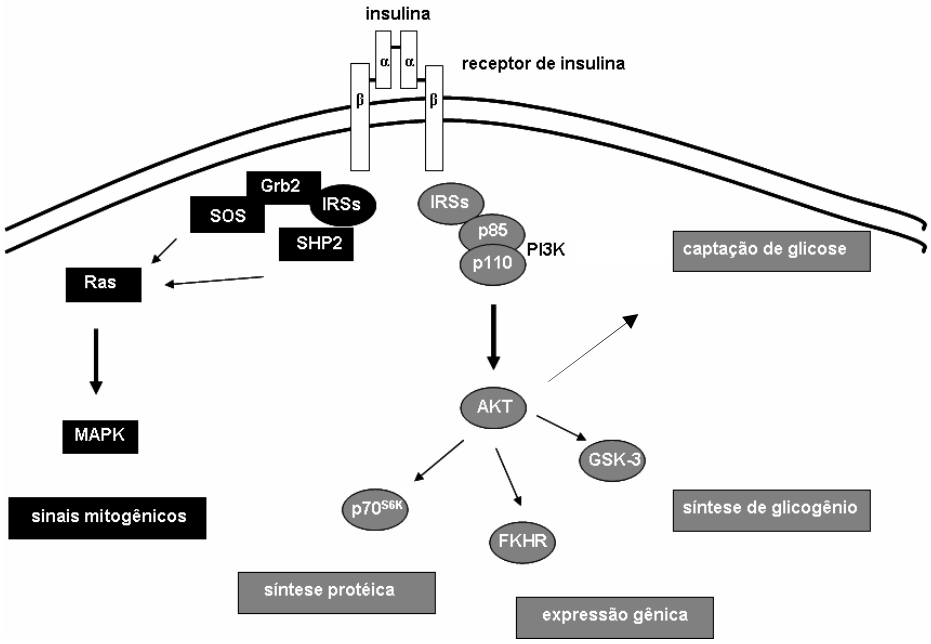

Figura 2 *Via de sinalização da insulina nas células* A insulina promove seus efeitos ligando-se ao seu receptor específico ativando a atividade tirosina cinase intrínseca deste. A cinase ativada do receptor de insulina fosforila substratos intracelulares como IRS, que por sua vez se liga a várias moléculas sinalizadoras contendo domínios SH2. Entre elas, o complexo Grb2-SOS e SHP-2 transmitem sinais mitogênicos através da ativação da Ras. Em contraste, a PI3K transmite as maiores ações metabólicas da insulina via efetores "downstream" como a Akt. . SOS – "son-of-sevenless"; Ras, proteína associada a GTPase; MAPK – proteína cinase ativada por mitógeno; GSK3 – cinase de glicogênio sintase 3

Ciclo Celular de Células β

Embora Swenne (1985) tenha demonstrado há mais de 2 décadas que as células β apresentavam a capacidade de se proliferar, até recentemente a maioria dos investigadores descartavam a hipótese de que células β maduras e diferenciadas fossem capazes de proliferarem, ou que o faziam muito raramente. Fenótipos restritos ao crescimento de células β foram observados através de estudos provenientes de grupos focados na biologia do desenvolvimento ou na oncologia. Após superexpressão ou deleção de determinado gene associado ao controle geral do ciclo celular esses pesquisadores conseguiram demonstrar alterações surpreendentes como hiperplasia de célula β pancreáticas (Hanahan, 1985; Rane *et al.*, 1999). Um dos motivos para se pressupor que células β não proliferavam estava no fato de que elas normalmente o faziam raramente de tal maneira que sua determinação era de difícil observação.

Finegood *et al.* (1995) estimaram a razão de proliferação de células β adultas em 3% a cada 24 h em camundongos e ratos. Proliferação de células β adultas menor que 0,07%/dia e de 0,04%/dia em camundongos (Teta *et al.*, 2005) e em humanos, respectivamente, foi demonstrado (Butler *et al.*, 2003). Entretanto, é sabido que a glicose pode induzir a proliferação de células β *in vitro* (revisado em Cozar-Castellano *et al.*, 2006). Ainda, durante a gestação e no período neonatal sabe-se que há aumento da proliferação de células β (Kassen *et al.*, 2000). Recentemente, Dor *et al.* (2004), usando métodos de rastreamento celular em pâncreas de camundongos, determinaram que apesar de baixa, a proliferação de células β desempenha um papel central na manutenção da massa deste tipo celular, e em reposição desta após pancreactomia parcial.

Um esquema simplificado dos componentes que regulam o ciclo celular em células β está demonstrado na Figura 3. A passagem da célula β pela fase S do ciclo celular exige a ativação e/ou repressão de inúmeros genes que serão indispensáveis para a formação das células-filhas. O controle e a sincronização destes genes ocorrem via uma família de ativadores e repressores transcricionais denominados de proteínas E2F (Cam & Dynlacht, 2003; Trimarch & Lees, 2001). As proteínas E2F 1, 2 e 3 estão associadas ao controle de inúmeros genes que levam a progressão do ciclo celular enquanto as proteínas E2F 4, 5 e 6 agem reprimindo genes supressores da progressão do ciclo celular. A proteína retinoblastoma (pRb) desempenha papel central como "freio" da progressão do ciclo celular forçando a parada deste na fase G_1/S (Berns 2003; Chau & Wang, 2003). A pRb inibe a progressão do ciclo celular de duas maneiras distintas: primeiro, a pRb se liga preferencialmente as proteínas E2Fs 1-3 reprimindo suas atividades trancripcionais. Segundo, a pRb força a parada do ciclo recrutando deacetilases de histonas que remodelam a cromatina e previnem o acesso dos fatores de transcrição aos seus promotores. A fosforilação da pRb em resíduos de serina e treonina inativa esta proteína liberando-a da associação com os E2Fs, permitindo que esses fatores de transcrição interajam com seus respectivos promotores. A fosforilação da pRb está sob o controle dos complexos ciclinas tipo D e de cinases dependentes de ciclinas 4 e 5 (Cdk-4 e Cdk-6, respectivamente). Camundongos "knock out" para Cdk-4 apresentam anormalidades em três tecidos, entre eles as células β pancreáticas, que apresentaram hipoplasia levando ao diabetes e cetoacidose (Rane *et al.*, 1999; Tsuitsui *et al.*, 1999). Superexpressão das proteínas Cdk-4 e ciclina D_1 em ilhotas de camundongos, ratos e humanos resulta em marcante fosforilação da pRb e aumento da proliferação de células β (Cozar-Castellano *et al.*, 2004; Cozar-Castellano *et al.*, 2006). Recentemente, Fatrai *et al.* (2006) demonstraram aumento da atividade da Cdk-4 em ilhotas pancreáticas com a proteínas Akt

constitutivamente ativa. Há três tipos de ciclinas: D_1, D_2 e D_3. As ciclinas D se associam a Cdk-4 ou Cdk-6 ativando a função cinase destas últimas, levando à fosforilação e inativação da pRb. Assim, agindo em concerto com as Cdk-4 ou Cdk-6 as ciclinas estimulam a progressão do ciclo celular. Superexpressão de ciclina D_1 em camundongos resulta em proliferação de células β (Zhang *et al.*, 2005). Recentemente, também foi demonstrado o papel crucial da ciclina D_2 sobre a massa e função das células β (Geórgia & Bhushan, 2004; Kushner *et al.*, 2005).

Duas famílias de proteínas apresentam modulação negativa sobre o complexo Cdk-4/-6/ciclinas D: são as cinases inibitórias (INKs) e proteínas inibitórias de ciclinas (CIPs) ou proteínas inibitórias de cinases (KIPs) (Figura 3). As INKs incluem as proteínas: $p15^{ink4b}$, $p16^{ink4a}$, $p18^{ink4c}$ e $p10^{ink4d}$. Entre as proteínas CIP/KIP incluem as proteínas: $p21^{cip}$, $p27^{kip}$ e $p57^{kip2}$. Ambas as INKs e CIPs/KIPs são amplamente expressas em diversos tecidos e inibem a progressão do ciclo celular ao se ligarem aos complexos Cdks, impedindo a atividade cinase destes (Pestell *et al.*., 1999; Pagano & Jackson, 2004). Ilhotas $p21^{-/-}$ são mais responsivas em termos de proliferação a fatores de crescimento comparada as ilhotas $p21^{+/+}$ (Cozar-Castellano *et al.*, 2006). Superexpressão da proteína p27 sob o controle do promotor da insulina em camundongos culmina em redução da proliferação destas, hipoplasia de ilhotas e cetoacidose (Uchida *et al.*, 2005). Assim, as proteínas INKs/CIPs/KIPs apresentam papel fundamental sob o controle da progressão do ciclo celular.

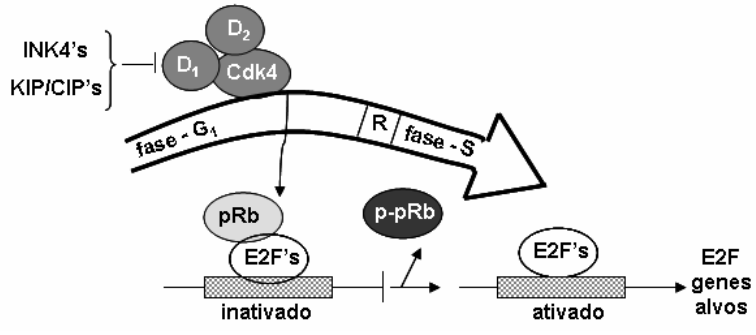

Figura 3 *Representação esquemática da célula β, mostrando os componentes que controlam a progressão da fase G_1 para S*. Consultar texto para detalhes. **D_1**, ciclina D_1; **D_2**, ciclina D_2; **INK4**, cinases inibitórias de Cdk4; **KIP**, proteínas inibitórias de cinases; **CIP**, proteínas inibitórias de ciclinas; **E2F**, fator de elongação 2.

Diabetes Mellitus

As duas principais formas de diabetes mellitus são o diabetes tipo 1 (DMT1) e o tipo 2 (DMT2) (ADA, 1997). Por diminuírem a qualidade e a expectativa de vida de milhões de indivíduos afetados, formam a maior causa de morbidade e mortalidade em seres humanos. Dentre inúmeras conseqüências, o diabetes mellitus pode causar doenças cardiovasculares, derrames cerebrais, cegueira, disfunções renais e amputações (Spellman, 2007). As estimativas são de que o DMT2 alcançará o número expressivo de 366 milhões de pessoas por volta do ano 2030 (Wild *et al.*, 2004). Disfunções na célula β como diminuição da síntese e/ou secreção de insulina e redução da massa deste tipo celular, devido ao aumento de apoptose e defeitos na regeneração, são componentes cruciais no desenvolvimento do diabetes mellitus (Eizirik & Mandrup-Poulsen, 2001; Mathis *et al.*, 2001; Rhodes, 2005).

O diabetes tipo 1 é caracterizado por redução severa na produção de insulina devido à destruição das células β pancreáticas que se desenvolve tipicamente nos primeiros anos de vida. Embora alguns biomarcadores relacionados ao sistema imunológico (por exemplo, autoanticorpos anti IA-2, GAD65 e insulina) possam identificar indivíduos de risco para o DMT1, o processo pelo qual células β são destruídas ainda não está bem compreendido. A perda de células β no DMT1 resulta de um processo mediado por auto-imunidade, onde a inflamação crônica conhecida como insulite causa destruição destas células. Este processo é mediado por citocinas e outros fatores secretados e/ou expressos pela superfície de células imunes que invadem as ilhotas e ativam vias secundárias de morte celular em alvos de células β (Eizirik & Mandrup-Poulsen, 2001; Gillespie, 2006; Mathis *et al.*, 2001).

Por outro lado, o DMT2, está frequentemente acompanhado por RI nos tecidos adiposo, muscular e hepático (Kahn *et al.*, 2006). Os mecanismos moleculares que

determinam a diminuição da massa de células β e a RI ainda não estão bem esclarecidos. O DMT2 resulta da incapacidade das células β pancreáticas secretarem quantidades de insulina suficientes em função da demanda metabólica requerida, ou seja, da utilização de glicose pelos tecidos periféricos (Kahn, 2001). Quando esse processo se prolonga, as células β podem se deteriorar e instalar-se um quadro metabólico denominado de intolerância à glicose que pode culminar no aumento progressivo dos níveis de glicose sanguínea e, eventualmente, hiperglicemia (Festa *et al.*, 2006; Weyer *et al.*, 1999; Cnop *et al.*, 2007). Defeitos tanto na secreção quanto na ação da insulina perifericamente contribuem para o desenvolvimento do DMT2, mas sabe-se que a deficiência de insulina é o componente crucial neste processo, sem o qual o DMT2 não se desenvolve. Este defeito secretório está presente no inicio da patogênese (Kahn, 2001) e é detectado por marcante redução da primeira da fase (também conhecida por fase aguda) da secreção de insulina estimulada por glicose (Cerasi *et al.*, 1995; Porte, 1991; Spellman, 2007).

O DMT2 não é herdado segundo as leis Mendelianas, uma vez que não há um grupo definido de genes que determina seu desenvolvimento. A presença de diversos genes tem sido identificada como fator de risco para o DMT2 (Shadek *et al.*, 2007). Os fatores genéticos que predispõem certos indivíduos obesos à falha de células β estão sendo identificados, embora seus mecanismos biológicos ainda não estejam claros (Grant *et al.*, 2006; Saxena *et al.*, 2007; Scott *et al.*, 2007; Sladek *et al.*, 2007). Assim, para reiterar, a presença de certos genes pode conferir risco ao DMT2, mas, ao que tudo indica, o maior fator que determina o desenvolvimento desta patologia é o estilo de vida, particularmente o excesso de consumo de calorias e o sedentarismo. Estudos *postmortem* têm mostrado que pacientes com DMT2 apresentam redução na massa de células β e aumento da taxa de apoptose destas células (Butler *et al.*, 2003, Clark *et al.*, 1988; Klöppel *et al.*, 1985; Sakuraba *et al.*, 2002; Yoon *et al.*,

2003). Entretanto, não está esclarecido se esta redução da massa é a única responsável pela diminuição da secreção de insulina no DMT2.

Resistência à Insulina

Embora se conheça grande parte dos mecanismos responsáveis pela diminuição da secreção de insulina e da massa de células β no DMT2, pouco se conhece acerca dos mecanismos compensatórios que ocorrem em ilhotas pancreáticas durante a RI. A RI geralmente está associada à obesidade e é uma condição que precede a instalação da hiperglicemia observada no DMT2 (Kasuga, 2006). A RI caracteriza-se pela diminuição da ação da insulina em tecidos periféricos (insulino-responsivos) como tecido adiposo, muscular e hepático. Estudos em culturas celulares têm revelado que a sinalização de insulina, que inclui o receptor de insulina, IRS, PI3-K, cinases dependentes de fosfatidil inositol cinase 1 (PDK1) e a proteína cinase serina/treonina Akt desempenham papel central nas ações metabólicas da insulina em inúmeros tipos celulares (Shepherd, 1998).

Camundongos transgênicos com deleção específica do receptor de insulina no fígado exibem RI, intolerância à glicose e ineficiência da insulina sobre a supressão da produção hepática de glicose bem como sobre a regulação da expressão gênica neste tecido (Michael *et al.*, 2000). Fenótipo similar foi demonstrado em camundongos em que a atividade da PI3K foi inibida especificamente no fígado como resultado da expressão desta isoforma mutada (dominante negativa) (Myiake *et al.*, 2002).

Resistência à Insulina e Glicocorticóides

Os glicocorticóides podem ser usados experimentalmente para indução de RI tanto *in vivo* (Saad *et al.* 1993; Rafacho *et al.*, 2008a; Severino *et al.* 2002; Nicod *et al.*, 2003) quanto *in vitro* (Burén *et al.* 2002; Ruzzin *et al.* 2005). Os efeitos dos glicocorticóides sobre tecidos periféricos insulino-dependentes como o tecido muscular, adiposo e hepático são bem conhecidos (Burén *et al.*, 2002, 2008; Ruzzin *et al.*, 2005; Saad *et al.*, 1995; Weinstein *et al.*, 1998). Ratos tratados com dexametasona (0.9 mg/kg por 2 dias) apresentam redução da captação de 2- [3^{H}]deoxiglicose em tecido muscular após estimulação por insulina (Weinstein *et al.*, 1998). A redução da sensibilidade à insulina em tecido muscular, proveniente de ratos tratados por 12 dias consecutivos com dexametasona, também foi constatada pela diminuição da captação de glicose induzida por insulina neste tecido que está associada com redução da fosforilação da proteína Akt induzida por insulina (Ruzzin *et al.*, 2005). Cultura primária de adipócitos provenientes de ratos, cultivados na presença de dexametasona por 24h, apresenta redução da captação de glicose basal bem como induzida por insulina, independente da concentração de glicose presente no meio de cultivo (Burén *et al.*, 2002). A RI em tecido hepático, muscular esquelético e adiposo observada em ratos tratados com dexametasona *in vivo* parece ser mediada por mecanismos pós-receptores. No primeiro tecido, foi observada diminuição da expresão e/ou fosforilação do receptor de insulina e do IRS-1 induzida pela insulina (Saad *et al.*, 1993), no músculo foi observado redução da fosforilação da Akt e da proteína glicogênio sintase cinase-3 (GSK-3) induzida pela insulina (Ruzzin *et al.*, 2005). Estudo recente de Burén *et al.* (2008) demonstra redução marcante tanto dos níveis de proteína quanto da fosforilação em Ser^{473} da proteína Akt em músculo e tecido adiposo de ratos submetidos a 11 dias de administração de dexametasona (1mg/kg/dia). Também

observaram redução da glicogênio sintase em adipócitos apenas. Assim, a indução de RI promovida pela administração de dexametasona é mediada principalmente pela redução da atividade da Akt tanto em músculo quanto gordura e pela redução da glicogênio sintase em tecido adiposo.

Mecanismos Compensatórios

Durante a RI, as ilhotas pancreáticas usualmente respondem com aumento da secreção de insulina para manutenção da normoglicemia, processo conhecido como compensação pelas células β. Os mecanismos envolvidos ainda não são bem compreendidos, mas é aparente (a partir de estudos com roedores) que tanto a expansão da massa de células β (Jetton *et al.*, 2005; Rafacho *et al.*, 2008b; Steil *et al.*, 2001) quanto o aumento da função destas células são importantes (Chen *et al.*, 1994; Liu *et al.*, 2002; Rafacho *et al.*, 2007, Rafacho *et al.*, 2008a,b) (Figura 4). A manutenção da massa de células β pancreáticas é fundamental para homeostase glicêmica, e sua diminuição está entre os maiores agravantes para o desequilíbrio da homeostase deste substrato.

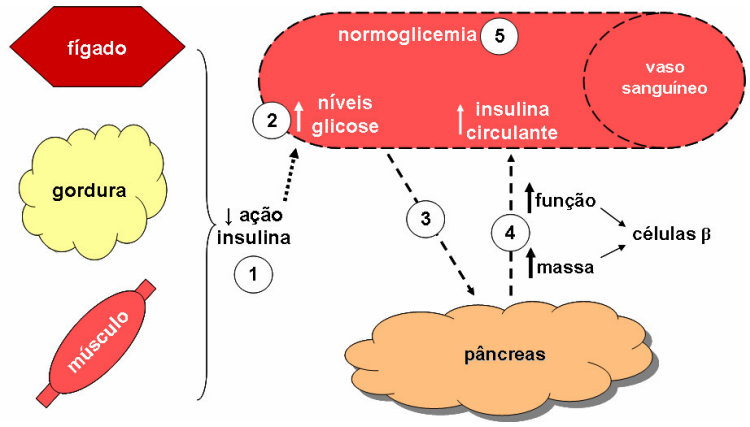

Figura 4 *"Feedback" entre ação periférica da insulina e pâncreas endócrino.* **(1)** redução da ação da insulina em tecidos periféricos (insulino-dependentes); **(2)** hiperglicemia transitória; **(3)** estimulação dos mecanismos compensatórios; (4) aumento da função (secreção de insulina) e estrutura (massa células β) do pâncreas endócrino; **(5)** normalização da glicemia à custa de hiperinsulinemia.

A massa de células β é mantida através do balanço entre apoptose e proliferação destas células (Bonner-Weir, 2000). Nos últimos anos, tem se tornado evidente que a proliferação de células β desempenha o maior papel no controle da massa de células β no indivíduo adulto (Dor *et al.*, 2004; Teta *et al.*, 2005; Teta *et al.*, 2007). Desta forma, há um grande interesse em identificar os reguladores endógenos da expansão da massa de células β.

A glicose tem sido sugerida como estímulo para a proliferação de células β (Bernard *et al.*, 1999; Bonner-Weir *et al.*, 1989; Paris *et al.*, 2003; Steil *et al.*, 2001; Topp *et al.*, 2004). Infusão de glicose 50% por curto período em camundongos constitui em modelo de hiperinsulinemia e hiperglicemia moderada. Estes camundongos apresentam aumento da proliferação sem alterações no tamanho e massa de células B (Alonso *et al.*, 2007). Adicionalmente, tem sido demonstrado que o metabolismo de glicose é indispensável para a proliferação compensatória de células B durante a resistência à insulina induzida por dieta hiperlipídica (Terauchi *et al.*, 2007).

Inúmeros pesquisadores têm reportado efeito de estimuladores sobre a proliferação de células B independentes de glicose, provavelmente porque o efeito da glicose é mediado via insulina (Muller *et al.*, 2006; Ogino *et al.*, 2006; Okada *et al.*, 2007). O aumento da atividade de caspase-9 e aumento de incorporação de BrdU, induzido pela glicose, é bloqueado pela adição de anticorpo anti-insulina ou pelo inibidor de PI3-K em células MIN6 (Muller *et al.*, 2006). A insulina promove a sobrevivência de células β em parte pela ativação de PDX-1, gene homeobox essencial para o desenvolvimento do pâncreas que tem sido ligado à proliferação no adulto (Kulkarni *et al.*, 2004). Camundongos deficientes em receptor de insulina em células β apresentam redução da massa destas células (Kulkarni *et al.*, 1999; Otani *et al.*, 2004). Por outro lado, deficiência do receptor de fator de crescimento semelhante a insulina-1 (IGF-1) em células β não resultam em redução da massa destas células (Kulkarni *et*

al., 2002). Dupla deficiência tanto do receptor de insulina quanto do receptor de IGF-1 demonstraram que, entre os 2 fatores de crescimento, a insulina desempenha papel dominante na regulação da massa de células β em camundongos adultos (Ueki *et al.*, 2006). Mais recentemente, foi demonstrado que camundongos deficientes em receptor de insulina em células β pancreáticas e submetidos à dieta hiperlipídica não apresentam aumento da massa destas células. Ainda, exibem proliferação reduzida destas células comparada ao grupo controle não transgênico (Okada *et al.*, 2007). Esses dados sugerem que o receptor de insulina desempenha papel essencial na compensação de células β durante a obesidade. Este estudo, entretanto, não mostra os efeitos do bloqueio em curto prazo e crônico da sinalização de insulina uma vez que os receptores de insulina estão ausentes desde antes do nascimento destes animais. Além do mais, estes experimentos do tipo "tudo ou nada" não asseguram que os efeitos seriam os mesmos em modelos patológicos.

Outras descobertas também apontam para a importância da sinalização da insulina no controle da massa de células β. Por exemplo, camundongos "*knock out"* para o substrato do receptor de insulina 2 (IRS-2) desenvolvem DMT2 devido a defeitos na massa de células β (Withers *et al.*, 1998). Os níveis de mRNA do receptor de insulina e de IRS-2 estão diminuídos em ilhotas de pacientes DMT2 (Gunton *et al.*, 2005). A ativação da Akt (Bernal-Mizrachi *et al.*, 2001) e da via Raf/Erk (Beith *et al.*, 2008) (vias *downstrean* da sinalização da insulina) são essenciais no controle da proliferação de células β pancreáticas.

OBJETIVOS

Gerais

Avaliar as alterações em parâmetros metabólicos periféricos (sensibilidade à glicose e à insulina) bem como em parâmetros funcionais (secreção de insulina) e estruturais (crescimento de células β) do pâncreas endócrino desenvolvidos em modelo farmacológico de resistência à insulina através da administração de três concentrações distintas de dexametasona.

Específicos

- Determinação do crescimento corpóreo e parâmetros sanguíneos (no jejum: glicose, insulina, corticosterona, proteínas e lipídios; alimentados: glicose, insulina e ácidos graxos não esterificados);

- Avaliação da sensibilidade periférica à glicose e à insulina através dos métodos de ipGTT e ipITT ;

- Avaliação da responsividade e sensibilidade de ilhotas frente a estímulos com concentrações crescentes de glicose;

- Avaliação da responsividade de ilhotas frente a estímulos de Cch, arginina, leucina e KCl;

- Determinação do crescimento de células B (tamanho, proliferação e morte celular);

- Determinação do conteúdo de proteínas envolvidas no controle da função e do crescimento de células β (Irs-2, PI3-K, Akt, p-Akt, $p70^{S6K}$, PDX-1, ciclina D_1, ciclina D_2, Cdk4, pRb, PCNA e Bcl-2).

Os resultados alcançados durante a realização deste trabalho de Tese estão apresentados a seguir sob a forma de três artigos:

Artigo 1

Functional Alterations in Endocrine Pancreas of Rats With Different Degrees of Dexamethasone-Induced Insulin Resistance. *Pancreas* 36:284-296, 2008.

Artigo 2

Increased Pancreatic Islet Mass is Accompanied by Activation of the Insulin Receptor Substrate-2/Serine-Threonine Kinase Pathway and Augmented Cyclin D2 Protein Levels in Insulin-Resistant Rats. *International Journal of Experimental Pathology* 89:264-275, 2008

Artigo 3

High Doses of Dexamethasone Induce Increased Beta-cell Proliferation in Pancreatic Rat Islets. *American Journal of Physiology Endocrinoly Metabolism*, 2009. **doi:10.1152/ajpendo.90931.2008**

ARTIGO 1

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Functional Alterations in Endocrine Pancreas of Rats With Different Degrees of Dexamethasone-Induced Insulin Resistance

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Objectives: We have analyzed the peripheral insulin and glucose sensitivity in vivo, and islet function ex vivo in rats with different degrees of insulin resistance induced by dexamethasone (DEX).

Methods: Dexamethasone, in the concentrations of 0.1 (DEX 0.1), 0.5 (DEX 0.5), and 1.0 mg/kg body weight (DEX 1.0) was administered daily, intraperitoneally, to adult Wistar rats for 5 days, whereas controls received saline.

Results: Dexamethasone treatment induced peripheral insulin resistance in a dose-dependent manner. At the end of the treatment, only DEX 1.0 rats showed significant increase of postabsorptive blood glucose and serum triglycerides, and nonesterified fatty acids levels. Incubation of pancreatic islets in increasing glucose concentrations (2.8–22 mM) led to an augmented insulin secretion in all DEX-treated rats. Leucine, carbachol, and high KCl concentrations induced the insulin release in DEX 0.5 and DEX 1.0, whereas arginine augmented secretion in all DEX-treated groups.

Conclusions: We demonstrate that in DEX 0.5 and, especially in DEX 0.1 groups, but not in DEX 1.0, the adaptations that occurred in the endocrine pancreas are able to counteract metabolic disorders (glucose intolerance and dyslipidemia). These animal models seem to be interesting approaches for the study of degrees of subjacent effects that may mediate type 2 diabetes (DEX 1.0) and islet function alterations, without collateral effects (DEX 0.1 and DEX 0.5).

Key Words: glucocorticoids, glucose and insulin sensitivity, insulin secretion, pancreatic islets, plasma lipids

(*Pancreas* 2008;36:284–293)

Glucocorticoids are widely used in clinical practice. However, these hormones can induce insulin resistance and, depending on the dose and time of treatment, can cause or aggravate type 2 diabetes, the predominant form of diabetes in humans. In support of this concept, glucocorticoids when used in excess have been observed to lead to diabetes in patients who have Cushing syndrome[1,2] and also in patients submitted to different kinds of organ transplantation.[3] Glucocorticoids exert their diabetogenic effect by decreasing glucose uptake by peripheral tissues and increasing hepatic glucose output. These effects are present in patients who exhibit insulin resistance or an already overt type 2 diabetes. Differences in several pathophysiological parameters have been observed in these disturbances, however. Insulin resistance associated with obesity[4,5] and pregnancy[6] or induced by glucocorticoid treatment,[7] is observed with postabsorptive normoglycemia or a moderate hyperglycemia together with hyperinsulinemia. Dexamethasone (DEX)-induced insulin resistance is generally associated with a compensatory increase in β-cell mass,[8] an increase in total pancreatic insulin content,[9] and augmented glucose-stimulated insulin secretion.[10] In contrast, in type 2 diabetes induced by glucocorticoids, a marked postabsorptive hyperglycemia is seen together with a reduction in serum insulin levels,[11] total islet insulin content, insulin messenger RNA expression,[12] and especially, diminished glucose-induced insulin release in vivo[13] and in vitro.[14] It is well known that islet function is reciprocally related to peripheral insulin sensitivity, in that insulin secretion is adaptively increased in insulin resistance.[15] Thus, the mechanism of insulin resistance and/or type 2 diabetes seems to reside in the function of the β cells. Induction of different degrees of insulin resistance by different concentrations of DEX treatment is an interesting tool for the investigation of the islet adaptations during this condition. Data in the literature regarding the effects of high DEX concentrations (ie, 1 mg/kg) on the endocrine pancreas and some metabolic parameters in rats are well established; however, it is difficult to ensure whether the altered circulating factors (increased serum glucose, insulin, triglycerides, nonesterified fatty acids [NEFA]) may signal changes in insulin action and on islet function. Thus, we believe that using lower doses of DEX to generate a model with a slight degree of insulin resistance and, possibly diminish these collateral circulating factors, would represent a better model for the investigation of alterations in islet function.

MATERIALS AND METHODS

Materials

Dexamethasone phosphate (Decadron) was from Ache (Campinas, SP, Brazil). Sodium pentobarbital (3% Hypnol) was from Cristália (Itapira, SP, Brazil). Human recombinant

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insulin (Biohulin N) was from Biobrás (Montes Claros, Brazil). Corticosterone kit was from MP Biomedicals (Orangeburg, NY). Enzymatic colorimetric assay for the quantification of NEFA was from Wako Chemicals USA, Inc (Richmond, Va), triglycerides (TG) and total cholesterol (T-chol) detection kits were from Roche Diagnostics (Mannheim, Germany), and total serum protein (T-protein) and serum albumin detection kits were from In Vitro Diagnostica (Itabira, Brazil). Dextrose, NaCl, KCl, $CaCl_2$, $MgCl_2$, $NaHCO_3$, KOH, and Na_2SO_4 were from Mallinckrodt Baker, Inc (Paris, France). Collagenase, *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid, albumin, L-arginine, L-leucine, carbachol, activated charcoal, and dextran were from Sigma (St Louis, Mo). Ethanol, methanol, chloroform, and phenol were from Synth (Diadema, Brazil).

Animals

Experiments were performed on 4 groups of male Wistar rats (3 months old). The rats were obtained from the University of Campinas Animal Breeding Center and were kept at 24°C on a 12-hour light/dark cycle. The rats had access to food and water ad libitum. The experiments with animals were approved by the Institutional São Paulo State University Committee for Ethics in Animal Experimentation.

DEX Treatment

The rats received daily intraperitoneal (IP) injection, between 7:30 and 8:30 AM, for 5 consecutive days. Rats were divided into 4 groups according to the amount of drug administered (mg/kg body weight [BW], dissolved in saline): DEX 0.1, DEX 0.5, DEX 1.0, and saline alone (control [CTL]).

Metabolic, Hormonal, and Biochemical Measurements

Body weight and food intake were measured from 2 days before the beginning of treatment to the euthanasia day. On the day after the last DEX administration, fasted (12–14 hours) rats had the blood collected from the tail to measurement of blood glucose levels with a glucometer ("one touch"; Johnson & Johnson). Immediately after, they were killed (by exposure to carbon dioxide followed by decapitation), and the blood was collected by posterior serum obtaining. Organs (listed in Table 2) were gently withdrawn, weighed, and photographed when necessary. The serum, obtained by centrifugation, was used to measure all the following parameters. Insulin levels were measured by radioimmunoassay (RIA), using a rabbit antirat insulin antibody and rat insulin as standard.[16] Corticosterone content was measured (in a separate group killed by direct decapitation) according to the manufacturer's directions by RIA. The NEFA, TG, and T-chol were determined by enzyme-linked immunosorbent assay according to the manufacturer's directions. Total serum protein and serum albumin were quantified by spectrophotometer according to the manufacturer's directions. Serum NEFA was also determined from fed animals as described above.

Liver Glycogen Measurements

Determination of hepatic glycogen was performed according to Lo et al,[17] with some modifications. Briefly, the liver samples (300-500 mg) were transferred to test tubes containing 30% KOH (wt/vol) and boiled for 1 hour until complete homogenization. Then, Na_2SO_4 was added, and the glycogen was precipitated with ethanol. The samples were centrifuged at 800*g* for 10 minutes, the supernatants are discarded, and the glycogen was dissolved in hot distilled water. Ethanol was added and the pellets, obtained after a second centrifugation, were dissolved in distilled water in a final volume of 20 mL. Glycogen content was measured by treating a fixed volume of samples with phenol reagent and H_2SO_4, absorbance was then read at 490 nm with a spectrophotometer.

Intraperitoneal Glucose Tolerance Test

On the day after the last DEX administration, independent groups of fasted (12–14 hours) rats were anesthetized with sodium pentobarbital (3% Hypnol, 1 mL/kg BW). After checking the absence of corneal and pedal reflexes, unchallenged samples (time 0) were obtained from rats' tails. Immediately, 50% glucose (2 g/kg BW, IP) was administered, and blood samples were collected at 30, 60, 90, and 120 minutes from the tail tip for determination of glucose and insulin concentrations. For insulin determination, 75 μL of blood sample was added to an Eppendorf tube containing 200 μL of saline (NaCl 0.9%), centrifuged at 15,000*g* and stored at −70°C for subsequent measurement of insulin content by RIA.

Intraperitoneal Insulin Tolerance Test

On the day after the last DEX administration, independent groups of fed rats were anesthetized as described previously. A sample of blood was collected from the tail tip for glucose measurement at time 0. Human recombinant insulin equivalent to 2 units/kg BW, was then injected IP. Further samples were collected at 5, 10, 15, 20, 25, and 30 minutes for blood glucose measurement. The constant rate for glucose disappearance (Kitt) was calculated from the slope of the regression line obtained with log-transformed glucose values between 0 and 30 minutes after insulin administration.

Isolation of Islet, Insulin Content, and Static and Dynamic Secretion Protocols

Islets were isolated by collagenase digestion of the pancreas as described,[18] with modifications.[19] Insulin contents of islets were determined after extraction in acid-ethanol solution (12 mM HCl in 70% ethanol). The islets were sonicated for 15 seconds, extracted overnight at 4°C, centrifuged for 10 minutes at 3000*g*, and the supernatant was frozen for analysis of insulin content by RIA. For static incubation, groups of 5 islets were first incubated for 1 hour at 37°C in a Krebs-bicarbonate buffer solution of the following composition (in mM): 115 NaCl, 5 KCl, 2.56 $CaCl_2$, 1 $MgCl_2$, 24 $NaHCO_3$, 15 *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid and 5.6 glucose, supplemented with 0.5% of bovine serum albumin and equilibrated with a mixture of 95% O_2:5% carbon dioxide, pH 7.4. The medium was then replaced with fresh buffer containing the following solutions: 2.8, 5.6, 8.3, 11.1, 16.7, and 22 mM glucose, 20 mM arginine, 20 mM leucine, 1 mM carbachol, and 50 mM KCl (in the experiments using 50 mM KCl, the concentration of NaCl

was proportionally decreased to maintain osmolarity) in the presence of 2.8 mM glucose in the medium, and further incubated for 1 hour. At the end of the incubation, the samples were stored at -20°C for subsequent measurement of insulin content by RIA. For analysis of dynamic insulin secretion, 20 freshly isolated islets were transferred to perfusion cameras and perifused with Krebs-bicarbonate buffer solution at a flow rate of 1 mL/min for 100 minutes. The effluent was collected every 2 minutes into tubes that were stored at −70°C for insulin RIA. Perifusion consisted of 3 consecutive periods: 50 minutes with 2.8 mM glucose, 30 minutes with 16.7 mM glucose, and finally 20 minutes with 2.8 mM glucose. Collection of the samples started from 30 minutes of perifusion.

Statistical Analysis

Results are expressed as the means ± SEM of the indicated number (n) of experiments. Analysis of variance (1-way analysis of variance) for unpaired groups followed by Tukey posttest was used for multiple comparisons of parametric data. Correlation between dependent variables among the 4 groups was applied and was considered as significant values at $r \geq 0.7$. The significance level adopted was $P < 0.05$.

RESULTS

Reduction of Body and Adrenal Weight in Insulin-Resistant Rats

No difference in the body weight of rats was observed among the 4 groups on day 1 of the experimental period (Table 1). On the third day, however, a significant reduction in body weight was noticed in DEX 0.5 and DEX 1.0 compared with CTL rats. An additional reduction in body weight occurred before the end of the treatment in these 2 groups (n = 10; $P < 0.05$). The DEX 0.1 showed a significant reduction of body weight on the fourth day compared with the CTL group (n = 10; $P < 0.05$). No significant differences were observed in body weight in the rats from the DEX 0.1, DEX 0.5, and DEX 1.0 groups. Figure 1 shows representative adrenal glands and the respective mean weight values (8.8 ± 0.4, 7.1 ± 0.3, 6.2 ± 0.2, and 6.6 ± 0.2 mg/100 g BW for CTL, DEX 0.1, DEX 0.5, and DEX 1.0, respectively; n = 10 glands, 5 rats). The glands from DEX 0.1, DEX 0.5, and DEX 1.0 rats were hypotrophied with a mass reduction of 18%, 28%, and 25% compared with CTL, respectively.

TABLE 1. Body Weight and Food Intake in DEX-Treated and CTL Groups

	Body Weight (g)		Food Intake (g/kg BW)	
	Before Treatment	After Treatment	Before Treatment	After Treatment
CTL	360 ± 8	372 ± 8	66.6 ± 1.1	61.3 ± 2.7
DEX 0.1	350 ± 12	333 ± 11*	69.6 ± 1.6	43.1 ± 2.6*
DEX 0.5	354 ± 3	319 ± 2*	63.1 ± 2.7	42.6 ± 2.7*
DEX 1.0	355 ± 9	312 ± 8*	67.4 ± 1.3	39.6 ± 2.6*

Values are means ± SEM.
*Significantly different versus CTL. $P < 0.05$; n = 10. Analysis of variance followed by Tukey posttest.

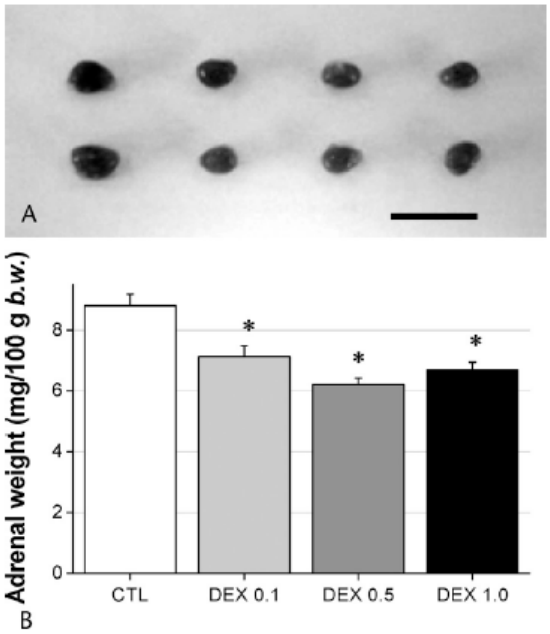

FIGURE 1. Exogenous glucocorticoid suppresses the corticosterone output. A, Adrenal glands photographed immediately after killing (representative). Note visible hypotrophy in both adrenals from DEX-treated rats. B, The columns illustrate the gland weights of all groups. Significant reduction occurred in the DEX 0.1, DEX 0.5, and DEX 1.0 groups. Data are means ± SEM *significantly different versus CTL. $P < 0.05$; n = 10. Analysis of variance with Tukey posttest. Bar = 10 mm.

Effects of Different Concentrations of DEX In Vivo on Food Intake and on the Overall Rat Organs

To explain the possible events that could be under the body weight reduction, we measured the food intake, along with the treatment, and the general and metabolic-specific organs' mass at the euthanasia day. At the third day of treatment, a significant reduction of food intake was observed in all DEX groups that persisted until the end period of treatment compared with CTL group (n = 10; $P < 0.05$) (Table 1). All DEX-treated rats showed a significant augmentation of liver, kidney, and heart mass (with exception in DEX 0.1 rats for heart) expressed as g/kg of BW compared with CTL rats (n = 10; $P < 0.05$) (Table 2). Retroperitoneal fat and spleen mass were reduced significantly in the 3 DEX groups versus CTL group (n = 10; $P < 0.05$). However, epididymal fat, extensor digitorum longus, and soleus muscles did not exhibit significant differences among all DEX and CTL groups. A 2.6- and 2.7-fold increase in hepatic glycogen content was observed in DEX 0.5 and DEX 1.0 groups compared with CTL ($P < 0.05$). The hepatic glycogen content was 2.1 ± 0.1, 2.7 ± 0.2, 5.5 ± 0.3, and 5.8 ± 0.3 mg/100 mg tissue for CTL, DEX 0.1, DEX 0.5, and DEX 1.0, respectively (n = 10).

TABLE 2. General and Metabolic-Specific Organ Mass in DEX-Treated and CTL Groups

(g/kg BW)	CTL	DEX 0.1	DEX 0.5	DEX 1.0
Liver	37.7 ± 1.2	40.1 ± 1.0	46.4 ± 1.2*	52.2 ± 1.6*†‡
Epididymal fat	13.3 ± 1.0	12.2 ± 0.8	15.4 ± 1.8	15.2 ± 1.6
Retroperitoneal fat	17.8 ± 1.2	11.0 ± 1.3*	11.8 ± 1.5*	10.2 ± 1.1*
EDL	0.47 ± 0.01	0.51 ± 0.02	0.51 ± 0.02	0.52 ± 0.01
Soleus	0.40 ± 0.01	0.45 ± 0.01	0.41 ± 0.03	0.44 ± 0.01
Heart	3.2 ± 0.06	3.4 ± 0.07	3.7 ± 0.13*	3.9 ± 0.1*†
Lung	5.5 ± 0.3	5.8 ± 0.3	5.8 ± 0.3	5.4 ± 0.2
Kidney	7.1 ± 0.1	7.4 ± 0.2	7.8 ± 0.3	8.8 ± 0.1*
Spleen	2.0 ± 0.08	1.6 ± 0.12*	1.3 ± 0.05*	1.3 ± 0.08*
Testicle	3.3 ± 0.09	3.1 ± 0.04	3.1 ± 0.05	3.0 ± 0.09

Values are means ± SEM.
*Significantly different versus CTL; †versus DEX 0.1; ‡versus DEX 0.5. $P < 0.05$; n = 10. Analysis of variance followed by Tukey posttest.
EDL indicates extensor digitorum longus.

Effects of Different DEX Concentrations on Blood Glucose and Serum Insulin, Corticosterone, T-Protein, Albumin, NEFA, TG, and T-chol Content

Table 3 shows that glucocorticoid treatment increased postabsorptive blood glucose levels in rats from the DEX 1.0 group (n = 10; $P < 0.05$ vs CTL). Although not significant, a marginal increase in blood glucose concentration was also observed with doses of 0.1 and 0.5 DEX. The postabsorptive serum insulin levels increased in a dose-dependent manner in all DEX-treated rats (n = 10; $P < 0.05$ vs CTL), indicating insulin resistance in these groups. No significant correlation was observed between the glucose and insulin levels, whereas a negative correlation was found between the final body weights with insulin values ($r = 0.97$, $P < 0.05$). The postabsorptive serum corticosterone levels were also reduced in a dose-dependent manner in all DEX groups versus CTL group (n = 10; $P < 0.05$), indicating an almost total suppression of endogenous corticosterone levels imposed by the exogenous glucocorticoid, especially in DEX 1.0 group. These serum corticosterone levels showed positive correlation with adrenal weights ($r = 0.96$, $P < 0.05$). Total serum protein was higher in DEX 1.0 rats compared with CTL group (n = 10; $P < 0.05$), whereas albumin levels were enhanced in DEX 0.5 and DEX 1.0 rats in a postabsorptive state (n = 10; $P < 0.05$) (Table 3). Total postabsorptive serum cholesterol level was not altered by DEX treatment. In contrast, DEX 0.5 and DEX 1.0 groups demonstrated a significant increase in serum TG levels compared with CTL (n = 5; $P < 0.05$), and these values correlated positively with serum insulin levels among the 4 groups ($r = 0.98$, $P < 0.05$). The DEX 1.0 group exhibited a significant increase in postabsorptive serum NEFA concentrations compared with CTL (n = 5; $P < 0.05$) (Table 4). A tendency toward an increase in NEFA concentration was observed in DEX 0.5 group, however, this increase was not statistically significant. Conversely, in absorptive state, both DEX 0.5 and DEX 1.0 groups showed a significant increase of serum NEFA levels (n = 10; $P < 0.05$).

Effect of DEX Treatment on Glucose Tolerance

The mean blood glucose levels during IP glucose tolerance test (IPGTT) were significantly higher in DEX 1.0 compared with CTL at all times (n = 10; $P < 0.05$). No differences were observed between DEX 0.1 and DEX 0.5 rats (Fig. 2A). The area under the IPGTT curve (AUC) was significantly higher only in DEX 1.0 group ($P < 0.05$ vs CTL) (Fig. 2B). With the exception of the 90-minute time point, insulin concentrations were significantly higher in DEX 1.0 than CTL during the entire experimental period (n = 10; $P < 0.05$) (Fig. 2C). Although not significantly different, the serum insulin levels in DEX 0.1 and DEX 0.5 groups were slightly higher after glucose loading than CTL until the end of the experimental period (n = 10; not significant). The insulin AUC was marginally higher in DEX 0.1 and DEX 0.5 and significantly higher in DEX 1.0 compared with CTL ($P < 0.05$) (Fig. 2D). These results suggest a clear glucose intolerance in DEX 1.0 group and a tendency toward glucose intolerance in DEX 0.5 rats.

TABLE 3. Blood Glucose and Serum Parameters Obtained in a Postabsorptive State in DEX-Treated and CTL Groups

	Glucose	Insulin	Corticosterone	T-chol	TG	T-protein	Albumin
	(mg/dL)	(ng/mL)		(mg/dL)		(mg/dL)	
CTL	98 ± 4.5	3.3 ± 0.2	89.3 ± 5.1	42.2 ± 5.2	75.1 ± 12.8	8.1 ± 0.1	3.4 ± 0.1
DEX 0.1	116.8 ± 5.2	9.7 ± 1.4*	44.7 ± 8.5*	34.9 ± 5.9	131.6 ± 8.7	8.3 ± 0.1	3.5 ± 0.3
DEX 0.5	118.1 ± 5.1	16.3 ± 1.7*†	19.2 ± 1.1*†	41.9 ± 4.7	225.6 ± 21.9*†	8.5 ± 0.1	3.9 ± 0.3*
DEX 1.0	135.2 ± 6.3*	17.3 ± 1.9*†	8.3 ± 1.4*†‡	40.4 ± 2.7	209.9 ± 17.6*†	8.9 ± 0.1*‡	4.0 ± 0.3*†

Values are means ± SEM.
*Significantly different versus CTL; †versus DEX 0.1; ‡versus DEX 0.5. $P < 0.05$. For glucose, insulin, corticosterone, T-protein, and albumin, n = 10; for TG and T-chol, n = 5. Analysis of variance followed by Tukey posttest.

TABLE 4. Serum NEFA Obtained in a Postabsorptive and Absorptive State in DEX-Treated and CTL Groups

NEFA (mM)	CTL	DEX 0.1	DEX 0.5	DEX 1.0
Postabsorptive	0.78 ± 0.08	0.62 ± 0.05	1.09 ± 0.12†	1.26 ± 0.08*†
Absorptive	0.37 ± 0.1	0.46 ± 0.1	0.71 ± 0.1*	0.80 ± 0.1*†

Values are means ± SEM.
*Significantly different versus CTL; †versus DEX 0.1.
$P < 0.05$; n = 5 for postabsorptive values and n = 10 for absorptive values. Analysis of variance followed by Tukey posttest.

Decreased Peripheral Insulin Sensitivity Induced by DEX Treatment

During IP insulin tolerance test, all DEX-treated rats exhibited insulin intolerance in a dose-dependent manner as shown by a significant reduction in the constant rate for glucose disappearance (Kitt) (n = 8; $P < 0.05$) (Fig. 3). The glucose disappearance rate was 1.4-, 2.8-, and 5-fold lower than that of the CTL for DEX 0.1, DEX 0.5, and DEX 1.0, respectively.

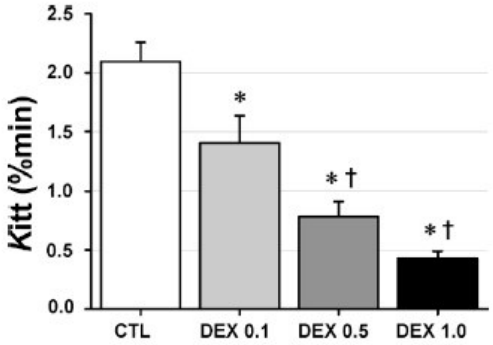

FIGURE 3. Insulin insensitivity in vivo is dependent on DEX concentration. Glucose disappearance rate measured in IP insulin tolerance test in CTL, DEX 0.1, DEX 0.5, and DEX 1.0 rats. A marked reduction in insulin sensitivity in DEX 1.0, and a smaller reduction in DEX 0.5 and DEX 0.1, was demonstrated from the constant rate for glucose disappearance (Kitt). Data are means ± SEM *significantly different versus CTL and †versus DEX 0.1. $P < 0.05$; n = 8. Analysis of variance with Tukey posttest.

DEX Treatment Increases Glucose and Non-Glucose–Induced Insulin Secretion in Isolated Islets

The total islet insulin content was not altered among all experimental groups, 212.8 ± 10.4, 212.3 ± 21.5, 218 ± 19.5, and 213.7 ± 17.4 ng/islet for CTL, DEX 0.1, DEX 0.5, and DEX 1.0, respectively. As shown in Figure 4A, at subthreshold glucose concentrations (2.8 mM), DEX 0.5 and DEX 1.0 islets showed augmented insulin secretion after normalization by the

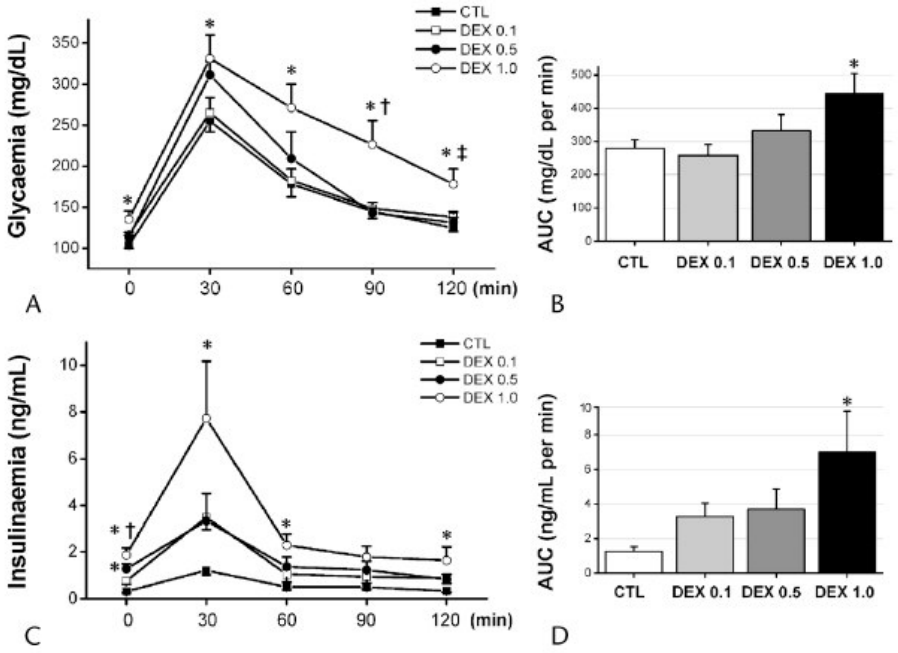

FIGURE 2. A high DEX dose promotes glucose intolerance. Glycemic and insulinemic profile obtained from IPGTT experiments in CTL, DEX 0.1, DEX 0.5, and DEX 1.0 rats. A, Even after 120 minutes of glucose load, DEX 1.0 rats demonstrated elevated glycemia compared with CTL. DEX 0.5 rats exhibited a tendency for diminished glucose tolerance as observed by glucose levels at 30 and 60 minutes, however, this decrease was not significant. The DEX 0.1 rats did not demonstrate alteration in glycemic profile in the IPGTT. C, Insulin peak is reached in all groups at 30 minutes, being significantly different in DEX 1.0 rats. Insulin values in DEX 0.1 and DEX 0.5 rats remained slightly but not significantly elevated. B and D, Integrated AUC data demonstrate a significant increment in DEX 1.0 rats for glucose and insulin values, respectively. Data are means ± SEM *significantly different versus CTL and †versus DEX 0.1 and ‡versus DEX 0.5. $P < 0.05$; n = 10. Analysis of variance with Tukey posttest.

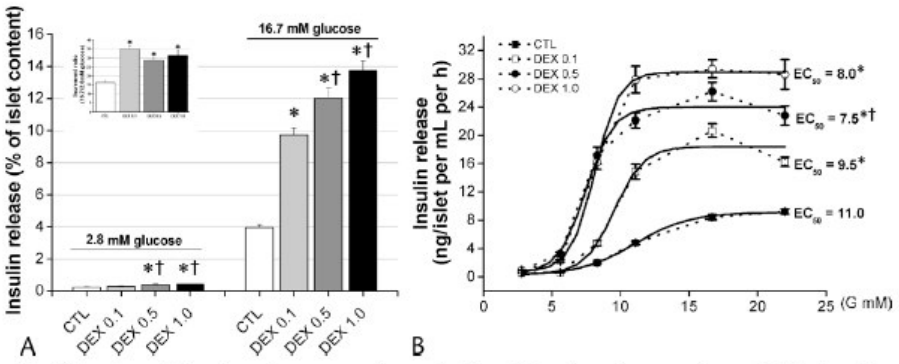

FIGURE 4. Glucocorticoid treatment, in vivo, increases glucose-induced insulin release and sensitivity. Insulin release from isolated islets of CTL, DEX 0.1, DEX 0.5, and DEX 1.0 rats. A, At a concentration of 2.8 mM glucose, insulin secretion was higher in DEX 0.5 and DEX 1.0 islets. At a stimulatory glucose concentration, the insulin secretion was enhanced in all rat groups treated with glucocorticoid (2.5-, 3-, and 3.5-fold higher than in CTL islets, to DEX 0.1, DEX 0.5, and DEX 1.0, respectively). Calculating the increment rate, in 2.8 mM to 16.7 mM glucose-induced insulin release, no difference among the 3 DEX-treated groups was observed (inset), however, rates were higher than in CTL. B, Dose-response curve obtained from insulin release by isolated islets' experiments. The dose-response curve to glucose (2.8–22 mM) was significantly shifted to the left in all DEX-treated rat islets ($P < 0.05$). Data are means ± SEM *significantly different versus CTL and †versus DEX 0.1. $P < 0.05$; n = 24. Analysis of variance with Tukey posttest.

total islet insulin content. At 16.7 mM of glucose, the insulin secretion was significantly increased in all glucocorticoid-treated groups (2.5-, 3-, and 3.5-fold higher for DEX 0.1, DEX 0.5, and DEX 1.0, respectively, compared with CTL; n = 24; $P < 0.05$). The insulin release rate between 2.8 mM and 16.7 mM increased in all DEX groups compared with CTL

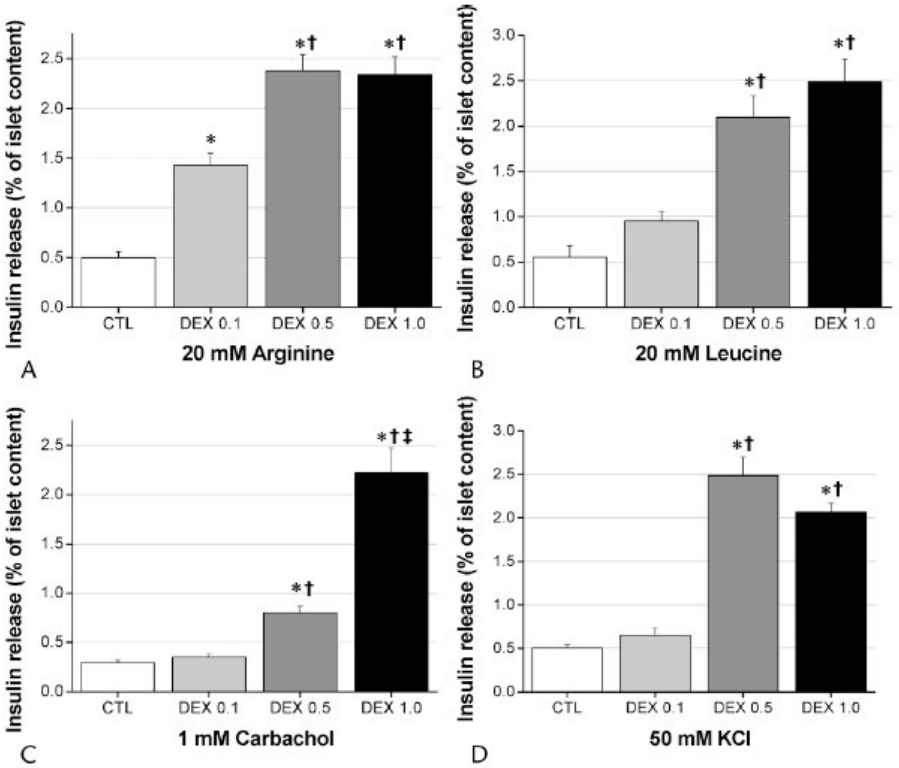

FIGURE 5. Arginine, leucine, carbachol, and KCl per se modulate insulin release in DEX islets. Insulin release from isolated islets of CTL, DEX 0.1, DEX 0.5, and DEX 1.0 rats. A, DEX 0.1, DEX 0.5, and DEX 1.0 islets responded to 20 mM arginine with increased insulin release. B, DEX 0.5 and DEX 1.0 exhibited a high insulin response to 20 mM of leucine (C) 1 mM carbachol and (D) 50 mM KCl. Data are means ± SEM *significantly different versus CTL, †versus DEX 0.1 and ‡versus DEX 0.5. $P < 0.05$; n = 12. Analysis of variance with Tukey posttest.

($P < 0.05$), but did not differ among the 3 DEX groups (inset of Fig. 4A). The dose-response curve to glucose (2.8–22 mM) was significantly shifted to the left in islets from all DEX-treated groups compared with CTL (Fig. 4B), with EC_{50} of 11.0, 9.5, 7.5, and 8.0 mM glucose for CTL, DEX 0.1, DEX 0.5, and DEX 1.0, respectively ($P < 0.05$).

Figure 5 shows that 20 mM arginine, 20 mM leucine, 1 mM carbachol, and 50 mM KCl significantly stimulated insulin secretion in islets from the 4 experimental groups compared with the secretion obtained in CTL islets of each respective group, incubated only in the presence of 2.8 mM glucose (n = 12; $P < 0.05$). The increase in insulin secretion induced by arginine was significantly higher in DEX 0.1, DEX 0.5, and DEX 1.0 islets compared with the increase observed in CTL islets; however, secretion was only significantly different in DEX 0.5 and DEX 1.0 for the other secretagogues.

Glucose-induced insulin secretion from perifused islets of DEX-treated and CTL rats is depicted in Figure 6. In the presence of nonstimulatory glucose concentrations (0–20 minutes), the islets from DEX 0.5 and DEX 1.0 groups secreted more insulin than CTL (n = 4; $P < 0.05$). A typical biphasic pattern of insulin secretion was observed in CTL islets after the introduction of 16.7 mM glucose in the perifusate (20–50 minutes) (see inset in Fig. 6). The islets from the DEX-treated rats (all groups) also showed a biphasic response to glucose with a first secretion peak followed by a sustained and progressively increased second phase. Insulin secretion in both phases is marginally higher in DEX 0.1 islets and significantly different in DEX 0.5 and DEX 1.0 than in CTL. The integrated insulin secretion, measured between 20 and 50 minutes of the perifusion period, was significantly higher in the DEX 0.5 and DEX 1.0 groups compared with the CTL (n = 4; $P < 0.05$). Finally, the insulin secretion in all groups of islets returned to basal values when the glucose concentrations were reduced to 2.8 mM glucose (50–70 minutes).

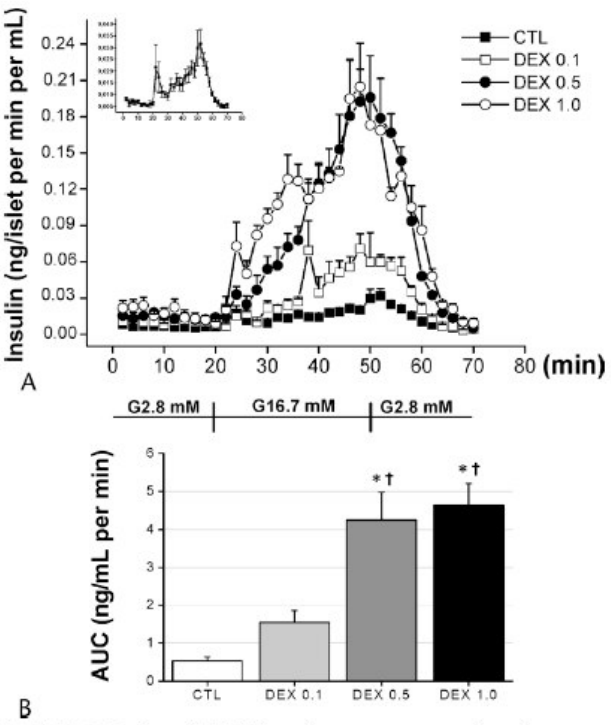

FIGURE 6. Perfused DEX islets demonstrate an altered insulin-output pattern. Glucose-stimulated insulin secretion from the isolated perifused islets of CTL, DEX 0.1, DEX 0.5, and DEX 1.0 rats. A, The islets from the DEX-treated rats (all groups) showed a biphasic response to glucose with a first secretion peak followed by a sustained and progressively increased second phase compared with those observed in CTL islets (see inset in detail). B, The AUC data revealed an increased insulin response to glucose in DEX 0.5 and DEX 1.0. Data are means ± SEM *significantly different versus CTL and †versus DEX 0.1. $P < 0.05$; n = 4. Analysis of variance with Tukey posttest.

DISCUSSION

We have assessed several physiological parameters in adult Wistar rats induced to different degrees of insulin resistance by administration of increasing doses of DEX for 5 consecutive days. Furthermore, the insulin secretion from isolated pancreatic islets, challenged by different stimulators, was also investigated. Basic biochemical parameters observed in the DEX 0.5 and DEX 1.0 groups are in agreement with those of glucocorticoid-induced insulin resistance and included alterations such as body weight reduction,[20] increased hepatic glucose output,[21] and increased plasma proteins levels.[22] In DEX 0.1, however, there were no alterations in liver glycogen content and plasma proteins levels. All DEX-treated groups showed reduction of food intake from the third day of treatment. It is well known that insulin exerts anorexigenic effects on the hypothalamus.[23] Insulin injection into the lateral cerebral ventricle of rats results in a reduction of 50% of food intake.[24] Conversely, during chronic administration of low DEX doses (0.1–4 μg, dissolved in drinking water), rats does not exhibit reduction of food intake. The body weight reduction in these animals, at least in part, is caused by increased lipolysis.[25,26] We speculate that the increased serum insulin level observed in our DEX-treated rats is the major component responsible for the body weight loss by acting on hypothalamus and favoring the suppression of food intake. Although we cannot rule out the probable increase of caloric expense in our DEX-treated rats, as judged by the reduction of retroperitoneal fat mass (which indicates substrate utilization to account for the full metabolism), other organs such as liver and kidney showed a gain of weight counteracting the absolute fat weight loss.

Our results are also in agreement with the observation that glucocorticoid-induced insulin resistance is accompanied by an increase in circulating insulin levels as a result of a compensatory response by pancreatic β cells,[9] that is, by a lower EC_{50} to glucose in insulin secretion. In healthy humans, changes in insulin sensitivity are normally compensated by reciprocal changes in glucose-stimulated insulin secretion in vivo, such that the product of insulin sensitivity and insulin

secretion remains unchanged.[27] This is reconfirmed in our DEX-treated rats, particularly in DEX 0.1 and DEX 0.5 rats. In these groups, the postabsorptive serum insulin levels were significantly increased and sufficient to prevent hyperglycemia. Furthermore, the insulin secretion response during the glucose tolerance test seems to be able to counteract the elevation in glucose concentration. Although moderate hyperinsulinemia was sufficient to prevent increases in blood glucose levels in DEX 0.1 and DEX 0.5 rats, a significant increase in circulating insulin did not efficiently maintain normoglycemia in DEX 1.0 rats. The glucose profile exhibited by DEX 1.0 after a glucose load demonstrates that supplemental insulin secretion was not enough to prevent the glucose intolerance shown by this group. These inadequacies in vivo, particularly with regard to the glucose intolerance observed in DEX 1.0 rats, are similar to those exhibited by 18- and 26-month-old rats[10] and transgenic mice with increased glucocorticoid sensitivity.[28] Novelli et al[10] showed that most of the 18- and all 26-month-old rats treated with 0.125 mg/kg of DEX for 13 days display higher plasma glucose levels than those considered as diabetic values, together with plasma insulin levels that are 4- to 5-fold higher than those of CTLs. In contrast, 3-month-old rats were able to prevent hyperglycemia with a 2-fold increase of plasma insulin levels, similar to those observed in our DEX 0.1 and DEX 0.5 rat groups. Chronic elevation of glucocorticoid signaling in transgenic mice with overexpression of glucocorticoid receptor in β cells also culminates in hyperglycemia and impairment of glucose tolerance.[28] Female rats submitted to a high-fat diet for 4 weeks and treated with DEX (0.1 mg/kg) for 5 days exhibit postabsorptive hyperinsulinemia, together with normoglycemic values; however, moderate glucose intolerance occurs because the glucose-stimulated insulin secretion after an intravenous bolus of glucose is not different from that of the CTL group. Thus, the insulin is not sufficient to prevent glucose increase.[4]

We have also demonstrated alterations in lipid metabolism, especially increases in TG and NEFA levels in both DEX 0.5 and DEX 1.0 groups. Evidence exists to suggest that glucocorticoids may increase serum TG levels, inhibiting lipoprotein lipase[26,29] and increasing NEFA by the activation of hormone-sensitive lipase[30] and by decreasing phosphoenolpyruvate carboxykinase activity in adipose tissue.[31] The increase in circulating insulin levels in DEX 0.1 rats seems to be efficient in maintaining its antilipolytic effect even in the presence of glucocorticoid. The increase in serum insulin concentrations, observed in DEX 0.5 and DEX 1.0 rats, however, was not enough to avoid hypertriglyceridemia and increased NEFA concentrations (NEFA levels only in absorptive period to DEX 0.5) in these groups. The idea that the increase in plasma NEFA concentrations, although contributing to the induction or aggravation of peripheral insulin resistance, could in parallel mediate insulin hypersecretion either directly[32–34] or by favoring TG synthesis in β cells and subsequent generation of lipid signaling molecules through lipolysis has been supported.[35] A recent study using Zucker fatty rats (ZF), a model of severe obesity, hyperlipidemia, hyperinsulinemia, and insulin resistance, demonstrated the importance of NEFA enhancement for insulin secretion in β-cell compensation for insulin resistance.[36] They showed that ZF islets are more sensitive to the effect of exogenous NEFA in augmenting glucose-stimulated insulin secretion. In addition, palmitate oxidation at 8 mM glucose and the net esterification of palmitate into total complex lipids into TG and diacylglycerol was higher in ZF islets compared with Zucker lean rats. The values of serum NEFA in DEX 0.5 and DEX 1.0 rats in absorptive state were higher than that of CTL rats and more elevated than those observed in ZF rats at the same nutritional period. Thus, it is possible that glucocorticoid-induced insulin resistance could have any modulation of NEFA on islet compensation for insulin resistance.

We also studied the secretory capacity of isolated islets derived from CTL and DEX-treated rats using static and dynamic incubations. After 1 hour of incubation at several glucose concentrations, we observed a greater insulin secretion in islets from the 3 DEX groups compared with CTL islets. These results suggest that the increase in insulin secretion observed in all DEX-treated groups, in response to suprathreshold glucose concentrations, implies some degree of islet adaptation, probably as a result of the impairment of insulin action at the periphery. In fact, islets from DEX-treated rats showed an increased glucose sensitivity shown by the lower EC_{50} compared with that of CTL islets. Islets derived from the DEX 0.5 and DEX 1.0 groups also secreted more insulin in response to arginine, leucine, carbachol, and depolarizing concentrations of K^+, indicating that the increased sensitivity to glucose, mentioned earlier, is present in these islets despite the stimulus used. It is known that glucose and leucine and, to a lesser extent, arginine[37] increase the adenosine triphosphate (ATP)–adenosine diphosphate (ADP) ratio after being metabolized, triggering a cascade of events that leads to an increase in insulin secretion. Because nonmetabolized stimulators also induced higher insulin secretion in DEX-treated islets, we cannot ascribe this potentiation solely to an increase in the metabolism of subtracts, rather that DEX treatment seems to improve (probably indirectly) several steps of the mechanism of insulin secretion. One of those steps is certainly related to Ca^{2+} handling by β cells because the potentiation of insulin secretion in islets from DEX 0.5 and DEX 1.0 rats responds efficiently to carbachol, which increases cytosolic Ca^{2+}.[38] The islets from DEX 0.5 and DEX 1.0 rats also secreted more insulin in response to depolarizing concentrations of K^+ that increases the rate of Ca^{2+} entry into the β cells, and to arginine that, in addition to its weak generation of ATP, also depolarize β cells.[39] Interestingly, islets from DEX 0.1 rats also secreted more insulin when challenged with arginine than islets stimulated by leucine, carbachol, and KCl, indicating that the concomitant generation of ATP and of depolarization of β cells by arginine is a good stimulator and demonstrating that the treatment with low doses of DEX already induces modifications in β cells that make these cells more responsive to glucose. Taken altogether, these results suggest that in islets from DEX-treated rats, Ca^{2+} handling and the activity of enzymes involved in the exocytotic process, including various Ca^{2+}-dependent protein kinases, could be implicated in the improvement of insulin secretion. The insulin output from the perifused CTL and DEX-treated islets exhibited a typical

biphasic response when challenged with stimulatory concentrations of glucose. In addition, all DEX-treated islets showed a higher insulin secretion in the first and second phase compared with CTL. The AUC data show a marginal increase in DEX 0.1 islets and a significant increase in insulin output in DEX 0.5 and DEX 1.0 with respect to the CTL values. In pancreatic β cells, the secretory granules are distributed into 2 pools that are distinguished by their release competence and/or proximity to the plasma membrane.[40] Most of the granules (95%–99%) belong to a nonreleasable pool that must undergo a series of ATP-, Ca^{2+}-, time-, and temperature-dependent reactions (collectively referred to as mobilization or priming) to gain release competence and involved in the formation of soluble *N*-ethylmaleimide–sensitive fusion attachment protein receptor complexes. These granules account for the second phase of glucose-induced insulin secretion.[41] Thus, the degree of islet adaptation required to surpass the harmful effect on insulin activity that is provoked by DEX could probably include alterations in the priming, docking, and replenishment of the insulin granules. Recently, we demonstrated an association between insulin hypersecretion during the insulin resistance and increase of connexin 36 protein expression. Because gap junction is essential for appropriate islet function, an improvement of pancreatic β cell communication and interaction could contribute to this performance exhibited by DEX islets.[42]

In conclusion, our data demonstrate that DEX-induced insulin resistance is a dose-dependent phenomenon. In the DEX 0.5 and especially DEX 0.1 groups, but not in DEX 1.0, the adaptations of the endocrine pancreas are able to counteract metabolic disorders (glucose intolerance and dyslipidemia). In vivo administration of DEX (independently of concentration) exerts an as yet unidentified effect, making the islets more sensitive to glucose and other secretagogues. In DEX 1.0 rats, the augmented insulin secretion was not sufficient to overcome the insulin resistance effects induced in vivo by the glucocorticoid. Thus, other factors must operate in the intact organism in opposition to glucocorticoid-induced augmentation in insulin release. The marked increase in TG and NEFA in the serum of DEX 1.0 rats may alter the cross talk between insulin sensitivity and insulin secretion in vivo. This alteration contributes to development of glucose intolerance and reproduces the transition from insulin resistance with islet compensation to type 2 diabetes. These animals seem to be interesting models for the study of degrees of subjacent effects that may mediate type 2 diabetes (DEX 1.0) and islet function alterations, without collateral effects (DEX 0.5 and especially DEX 0.1).

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ARTIGO 2

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ORIGINAL ARTICLE

Increased pancreatic islet mass is accompanied by activation of the insulin receptor substrate-2/serine–threonine kinase pathway and augmented cyclin D_2 protein levels in insulin-resistant rats

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Summary

It is well known that glucocorticoids induce peripheral insulin resistance in rodents and humans. Here, we investigated the structural and ultrastructural modifications, as well as the proteins involved in beta-cell function and proliferation, in islets from insulin-resistant rats. Adult male Wistar rats were made insulin resistant by daily administration of dexamethasone (DEX; 1mg/kg, i.p.) for five consecutive days, whilst control (CTL) rats received saline alone. Structure analyses showed a marked hypertrophy of DEX islets with an increase of 1.7-fold in islet mass and of 1.6-fold in islet density compared with CTL islets ($P < 0.05$). Ultrastructural evaluation of islets revealed an increased amount of secreting organelles, such as endoplasmic reticulum and Golgi apparatus in DEX islets. Mitotic figures were observed in DEX islets at structural and ultrastructural levels. Beta-cell proliferation, evaluated at the immunohistochemical level using anti-PCNA (proliferating cell nuclear antigen), showed an increase in pancreatic beta-cell proliferation of 6.4-fold in DEX islets compared with CTL islets ($P < 0.0001$). Increases in insulin receptor substrate-2 (IRS-2), phosphorylated-serine-threonine kinase AKT (p-AKT), cyclin D_2 and a decrease in retinoblastoma protein (pRb) levels were observed in DEX islets compared with CTL islets ($P < 0.05$). Therefore, during the development of insulin resistance, the endocrine pancreas adapts itself increasing beta-cell mass and proliferation, resulting in an amelioration of the functions. The potential mechanisms that underlie these events involve the activation of the IRS-2/AKT pathway and activation of the cell cycle, mediated by cyclin D_2. These adaptations permit the maintenance of glycaemia at near-physiological ranges.

Keywords
cell cycle proteins, glucocorticoid, insulin resistance, pancreatic islet, structure, ultrastructure

Insulin resistance is associated with the pathogenesis of *diabetes mellitus*, which constitutes one of the main threats to human health (Anderson *et al.* 2003). The knowledge of the morphological and functional mechanisms that accompany insulin resistance is important, as this may aid in the development of future preventive therapies. The capacity of dexamethasone (DEX) to induce peripheral insulin resistance *in vivo* (Saad *et al.* 1993; Barbera *et al.* 2001; Severino *et al.* 2002; Nicod *et al.* 2003) and *in vitro* (Burén *et al.* 2002; Ruzzin *et al.* 2005) has been previously demonstrated and, depending on the dose and time of treatment, can cause type 2 diabetes (Pagano *et al.* 1983; Beard *et al.* 1984; Hoogwerf & Danese 1999). During the insulin resistance state, alterations are observed in the glucose metabolism in peripheral tissues such as liver, muscle and adipose tissues (as a result of the failure of these tissues to respond to insulin). These alterations were accompanied by functional and morphological changes in pancreatic beta cells. Peripheral insulin resistance provokes increased plasma insulin levels as a consequence of oversecretion of insulin by pancreatic islets in an attempt to keep the glycaemia close to physiological ranges (Barbera *et al.* 2001; Severino *et al.* 2002; Nicod *et al.* 2003). Insulin resistance also induces an increase in total pancreatic insulin content (Bonner-Weir *et al.* 1981) and a higher glucose-stimulated insulin secretion (GSIS) by islets *ex vivo* (Novelli *et al.* 1999; Holness *et al.* 2005). Compensatory islet hypertrophy towards chronic glucocorticoid treatment *in vivo* (Visser *et al.* 1979; Tomita *et al.* 1984; Zwicker & Eyster 1993) and in animal models that exhibit insulin resistance (Ogawa *et al.* 1992; Pick *et al.* 1998) has been described as the main morphological adaptation in this condition. The pancreatic beta-cell mass results from a dynamic balance of neogenesis, proliferation, cell volume changes and cell death (Bonner-Weir 2000, 2001). However, the molecular mechanisms involved in the regulation of beta-cell mass are not yet completely elucidated. Increased expression or activity of proteins related to the islet function and to the control of G_1/S cell cycle progression, such as insulin receptor substrate-2 (IRS-2), serine-threonine kinase AKT, cyclin D_1, cyclin D_2, cyclin-dependent-kinase-4 (CDK4) and p21 have been associated with augmented islet mass (Cozar-Castellano *et al.* 2004; Zhang *et al.* 2005; Fatrai *et al.* 2006; Niessen 2006; Fernández *et al.* 2006; Terauchi *et al.* 2007).

Recently, using different doses of DEX administration *in vivo*, it was demonstrated that the dose of 1.0 mg/kg induces marked peripheral insulin resistance and decreased glucose tolerance (Rafacho *et al.* 2008). Islets from these animals exhibited adaptive compensations, including increases in glucose- and other secretagogue-induced insulin secretion. The structural and ultrastructural data on pancreatic islets, referring to glucocorticoid-induced insulin resistance, are scarce; however, the majority of these studies only demonstrate islet hypertrophy as a compensatory adaptation to peripheral insulin resistance (Boquist 1972; Jonas *et al.* 1983; Tomita *et al.* 1984). Unfortunately, other studies employing prolonged periods of treatment with glucocorticoids refer to pancreatic islet observations in relation to diabetic conditions (Visser *et al.* 1979; Diani *et al.* 1987; Zwicker & Eyster 1993; Momose *et al.* 2006). Thus, the current study sought to investigate the structure and ultrastructure of pancreatic rat islets, as well as some target proteins involved in the function and control of beta-cell cycle progression in insulin-resistant rats.

Materials and methods

Animals

Experiments with animals were approved by the institutional (UNESP) Committee for Ethics in Animal Experimentation and conform to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996). Groups of five male Wistar rats (3 months old) from the breeding colony at UNESP were kept at 24 °C on a 12h light/dark cycle. The rats had free access to food and water. DEX-treated rats received daily injections of DEX (1mg/kg b.w., i.p., Decadron®; Aché, Campinas, Brazil) for 5 days, whereas control (CTL) rats received saline. On the day following the last DEX administration, fed rats were killed by exposure to CO_2, followed by decapitation. The blood was collected and immediately centrifuged. The serum insulin levels were detected by radioimmunoassay (RIA), utilizing guinea-pig anti-rat insulin antibody and rat insulin as standard (Scott *et al.* 1981).

Intraperitoneal insulin tolerance test (ipITT)

The ipITT was performed in separate groups as described in detail previously (Rafacho *et al.* 2007, 2008).

Structural and quantitative approaches

To study the morphological aspects and islet mass of endocrine pancreas, five pancreases from each group were excised, cleared of fat and lymph nodes, weighed, immersion-fixed for 12 h in Bouin's fixative solution, dehydrated and embedded in paraffin. At 250 μm intervals, four serial sections (5 μm) were cut on a rotary microtome and adhered to individual normal or silanized glass. The first section from each series

was stained with Gömori's trichrome to perform the morphological and stereological analysis. The second, third and fourth sections were immunoperoxidase stained for insulin, glucagon and proliferating cell nuclear antigen (PCNA), respectively, to qualify the distribution of pancreatic beta and alpha cells and the presence of proliferation in beta cells.

Islet mass. This was determined by point counting stereology (Weibel 1972) on Gömori's trichrome-stained sections. Each section was counted systematically with a grid of 100 points (final magnification ×320). The numbers of points over endocrine, exocrine and non-exocrine pancreatic tissue were counted. The relative islet volume was calculated by dividing the number of points over the endocrine tissue by the number of points over the total tissue. Islet mass was determined by multiplying the relative volume by the total weight of the pancreas. A minimum of 500 fields per rat was counted. The islet images for documentation were registered by a CCD camera, coupled to an Olympus BX-60 photomicroscope (Olympus, Tokyo, Japan).

Immunostaining. Cellular distribution of insulin, glucagon and PCNA was analysed using a standard indirect immunoperoxidase method. After paraffin removal, the sections were rehydrated and blocked against endogenous peroxidase activity with 1% H_2O_2. After washing with 0.01 M phosphate-buffered solution (PBS, pH 7.4) the sections were treated with 0.01 M sodium citrate buffer (0.05% Tween 20, pH 6.0) at 98 °C for antigen retrieval. Sections were then incubated for 30 min with PBS (0.05% Tween 20 and 5% of dry skimmed milk) followed by primary antibody incubation for 2 h at room temperature (RT). The antibodies used were rabbit anti-insulin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), goat anti-glucagon (Santa Cruz biotechnology) and rabbit anti-PCNA (Dako Cytomation, Carpinteria, CA, USA) diluted at 1:150, 1:75 and 1:150 in PBS with 2% of dry skimmed milk respectively. After washes in PBS, sections were incubated at RT for 30 min with LSAB (Dako Cytomation) for insulin or PCNA or with specific biotinylated secondary antibody (Dako Cytomation) for glucagon. Sections were then treated with horseradish peroxidase (HRP)-streptavidin solution from LSAB for insulin or AB system (Dako Cytomation) for glucagon during 30 min at RT. The streptavidin–biotin complexes were detected with diaminobenzidine (DAB) solution (0.1% DAB and 0.02% H_2O_2 in PBS). Finally, the sections were rapidly stained with Harris' haematoxylin and mounted for microscopic observation.

Islet and nucleus area, perimeter and roundness-factor. Pools of 1000 islets, isolated by collagenase digestion of the pancreas according to a previously published protocol (Rafacho *et al.* 2007, 2008), were collected with a Pasteur pipette and immediately immersion-fixed in 4% paraformaldehyde. Three pools of 1000 islets of each group were used to perform the measurement of total islet area and three other pools were used to determine the nucleus area. For total islet area the islets were submitted to Feulgen's DNA method *en bloc* (Mello 1997) and to determine nucleus area the islets were embedded in historesin (Leica, Wetzlar, Germany), sectioned at 3 μm and submitted to Feulgen's DNA method for posterior analysis. At least 500 islets and 300 nuclei for each group were randomly acquired by a CCD camera. The area (μm^2) and perimeter (μm) values were automatically obtained by the Image-Pro-Plus ® Media, Cybernetics program, coupled to an Olympus BX-60 photomicroscope. For islet measurements, perimeter values were used to calculate the roundness-factor and the following formula was used [$4\pi \cdot area/(perimeter)^2$]; for those considered to form a perfect circle, the resulting value was ≥1, and for islets that were not perfect circles, the resulting value was <1.

Beta-cell proliferation. Average beta-cell proliferation was obtained by counting total islet-cell nuclei stained for insulin and PCNA, using the software cited above. At least 40 islets (4800 ± 233 beta-cell nuclei) per animal were sampled. The beta-cell proliferation was estimated by the percentage of PCNA-positive cells of the total insulin-positive cells (Terauchi *et al.* 2007).

Transmission electron microscopy

Pools of isolated islets from the experimental groups were processed for transmission electron microscopy, as described previously (De Carvalho *et al.* 1994), employing the fixation procedure according to Cotta-Pereira *et al.* (1976). Briefly, isolated islets were fixed in 3% glutaraldehyde/0.25% tannic acid in Millonig's buffer pH 7.3 for 2 h, postfixed in 1% osmium tetroxide, dehydrated in acetone and finally embedded in araldite resin. After the selection of the regions of interest by trimming the material with a glass knife, ultrathin sections (50–75 nm) obtained with a diamond knife were collected and stained by uranyl acetate and lead citrate. Observation and electron micrographs were made with a LEO–Zeiss 906 transmission electron microscope (Eching bei München, Germany).

Insulin content and secretion

Insulin content and secretion were measured as described in detail previously (Rafacho *et al.* 2007, 2008). Briefly, after

islet isolation, groups of five islets were first incubated for 1 h at 37 °C in a Krebs-bicarbonate buffer solution containing 5.6mmol/l glucose, supplemented with 0.5% of bovine serum albumin and equilibrated with a mixture of 95% O_2:5% CO_2, pH 7.4. The medium was then replaced by fresh buffer containing 2.8 or 16.7 mmol/l glucose and incubated for a further 1 h. At the end of the incubation, the supernatant was collected and appropriately stored at −20 °C for subsequent measurement of insulin content by RIA, as described above.

Protein extraction and immunoblotting

The following antibodies were used: anti-insulin receptor subtrate-2 (IRS-2) (Cell Signaling, Beverly, MA, USA), anti-alpha-tubulin (used as a housekeeping antibody), anti-AKT, anti-phospho-AKT (ser473) (p-AKT) (Santa Cruz Biotechnology), anti-cyclin D_2 (Lab Vision, Fremont, CA, USA), anti-retinoblastoma protein (pRb) (BD Bioscience, Mississauga, ON, CA) and anti-PCNA (Dako Cytomation). Pools of islets were homogenized in ice-cold cell lysis buffer (Cell Signaling) using a cell homogenizer (Fisher Scientific, Suwanee, GA, USA) for 10 s at the maximum speed. Protein concentration from total cell lysate was determined by the RCDC method, according to the manufacturer (Bio-Rad, Hercules, CA, USA). Immunoblotting experiments were performed at least six times. Protein obtained from islets (100 μg) was used for each experiment. After blocking at RT for 2 h in Tris buffer salt tween (TBST)/5% dry skimmed milk, membranes containing islet lysates were washed in TBST (3 × 7 min) and incubated overnight with primary antibodies at the dilutions recommended by the manufacturers in TBST/3% dry skimmed milk. After washing in TBST (3 × 10 min), membranes were incubated with the appropriate secondary antibody conjugated with HRP for 90 min in TBST/1% dried skimmed milk at RT. Antibody binding was detected by enhanced SuperSignal® West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA), as described by the manufacturer. Blots were scanned (Epson expression 1600) and the densitometry of protein bands was determined by pixels intensity using Scion Image software (Scion Corporation, Frederick, MD, USA).

Statistical analysis

Results are expressed as the mean ± SEM of the indicated number (n) of experiments. Statistical comparisons between data from DEX and CTL groups were performed using the unpaired Student's t-test. The significance level adopted was $P < 0.05$.

Results

Development of insulin resistance

Increases in serum insulin levels and GSIS are features of insulin-resistant rodents (Weir *et al.* 2001). Previous works from our group have demonstrated that rats receiving 1.0 mg/kg DEX showed decreased insulin sensitivity, accompanied by a decrease in glucose tolerance. Increases in circulating serum insulin levels and GSIS were also observed in these rats (Rafacho *et al.* 2007, 2008). Herein, we analysed serum insulin, ipITT and GSIS parameters to confirm the presence of insulin resistance. DEX rats showed a marked increase in fed serum insulin values (25.8 ± 4.2 *vs.* 4.3 ± 0.6 ng/ml for DEX and CTL rats respectively; $n = 10$, $P < 0.05$). During ipITT, DEX rats exhibited decreased insulin sensitivity as shown by a significant reduction in the constant glucose disappearance rate that was threefold lower than that of the CTL ($n = 8$, $P < 0.05$). DEX islets showed a 3.4-fold increase in insulin secretion in islets incubated with 16.7mM glucose, compared to CTL islets (3.82 ± 0.22 *vs.* 13.22 ± 1.02% of islet content for CTL and DEX rats, respectively; $n = 12$, $P < 0.05$).

Light microscopic findings in pancreatic islets of DEX rats

Figure 1(a) shows a panoramic view of a histological section of pancreas from CTL rats. No major histological differences of the exocrine tissue were observed in pancreas from DEX-treated rats. However, varying degrees of islet hyperplasia were more often exhibited by the DEX pancreas (Figure 1c,d). Higher numbers of irregular islets, compared with the rounded and oval islets, were observed in DEX, compared with CTL pancreas (Figure 1c,d). The signs of hyperplasia are generally evidenced by coalescence of adjacent islets (Figure 1c) and enlargement and increased cellularity (Figure 1d). Although mitosis figures are a scarce phenomenon, they were more frequent in DEX, compared with CTL islets (compare Figure 1b with Figure 1e–g).

In CTL rats, beta-cells represent almost all the cells in the islets. The insulin-positive cells occupied the core and mantle of the islet (Figure 2a). Glucagon-positive cells were limited to the ring surrounding the islet (Figure 2b). The pattern of insulin-positive cell distribution in DEX islets was similar to that of CTL islets (Figure 2c). However, there was an apparent discontinuous ring of glucagon-positive cells in DEX islets compared with CTL islets (Figure 2d).

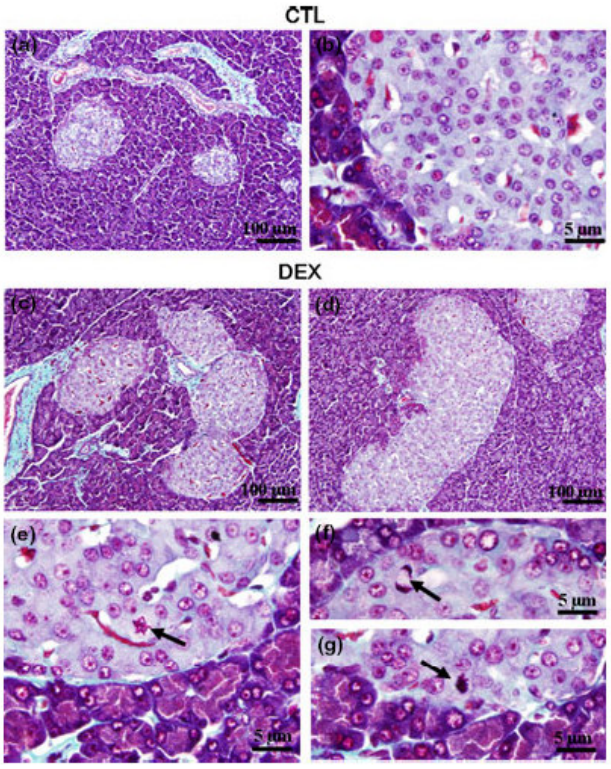

Figure 1 Morphological aspects of pancreatic islets from DEX and CTL rats. Islets of CTL (a, b) and DEX (c, d) rats. Arrows point to mitotic figures in DEX pancreas (e, g). Light microscope with Gömori's trichrome staining. Magnification ×200 in (a), (c) and (d) and ×1000 in (b), (e), (f) and (g).

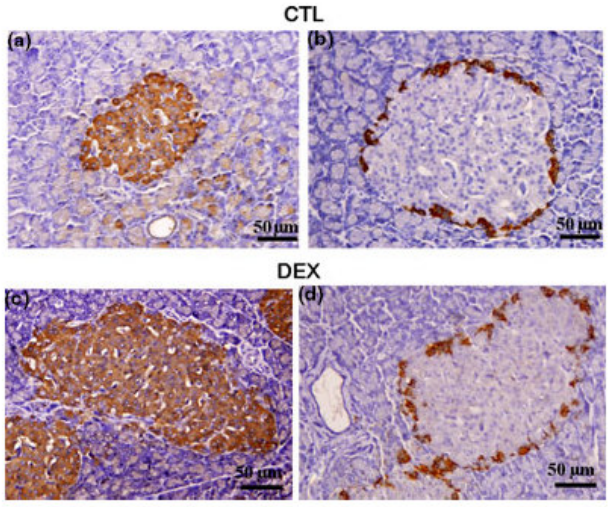

Figure 2 Cellular distribution of insulin- and glucagon-positive cells in pancreatic islets of DEX and CTL rats. CTL and DEX pancreas immunostained for insulin (a, c) and glucagon (b, d) respectively. Light microscope. Magnification ×400.

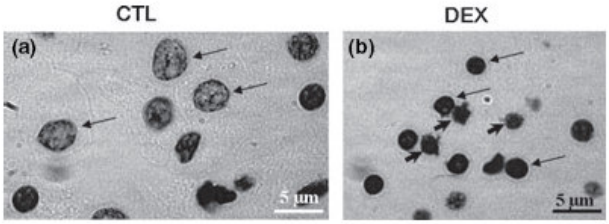

Figure 3 Nucleus of isolated islets stained by Feulgen's DNA method. Nuclei from CTL (a – arrows) and DEX (b – arrows) islet cells. Occasionally, pyknosis signals and bleb formation indicate the presence of suspect apoptotic phenotypes in DEX islet cells (b – arrowheads). See text for details of morphometrical data. Magnification ×1000.

Structural aspects of islet nuclei in DEX rats

Using Feulgen's DNA method, we observed a well-distributed uniform chromatin, characteristic of normal nuclei, in CTL islet cells (Figure 3a). However, some nuclei from DEX islet cells exhibited a more condensed chromatin compared with CTL islets (Figure 3b). Occasionally, pyknosis signals and bleb formation were observed in DEX-islet cells, indicating the presence of suspect apoptotic phenotypes (arrowheads in Figure 3b). The mean nuclei area and perimeter values were significantly lower in DEX, compared with those observed in CTL islet cells ($n = 300$, $P < 0.05$; Table 1). DEX islet cell nuclei demonstrated 32% and 20% reductions in area and perimeter values, respectively, compared with CTL.

Transmission electron microscopy assays

Ultrastructural evaluation of isolated DEX and CTL islets confirmed light microscopy results. Pancreatic CTL islet cells (alpha and beta) were easily identified by their characteristic secretory granules (Figure 4b). The secretory granules from pancreatic beta cells featured an electron-dense core and a translucent halo appearance (Figure 4d,e). In DEX islets, alpha and beta cells were also easily distinguishable (Figure 4g). However, DEX beta cells showed an increase in the number of secreting organelles, such as endoplasmic reticulum and Golgi apparatus. The endoplasmic reticulum was preferentially located at the cytoplasm periphery (Figure 4i,k) and the Golgi apparatus at the para-nuclear region (Figure 4h). Mitosis figures were confirmed at the ultrastructural level in DEX beta cells (Figure 4j).

Table 1 Area and perimeter values from control (CTL) and dexamethasone-treated (DEX) rat islets

	Area (μm2)		Perimeter (μm)		Roundness-factor	
	Islet	Nucleus	Islet	Nucleus	Islet	Nucleus
CTL	12,153 ± 420	30.5 ± 0.6	432 ± 7	21.5 ± 0.2	0.75 ± 0.008	0.83 ± 0.01
DEX	26,057 ± 915*	20.5 ± 0.5*	672 ± 15*	17.2 ± 0.2*	0.69 ± 0.008*	0.86 ± 0.06*

Values are mean ± SEM.
*Significantly different *vs.* CTL. $n = 500$ for islet and 300 for nucleus. $P < 0.05$. Unpaired Student's *t*-test.

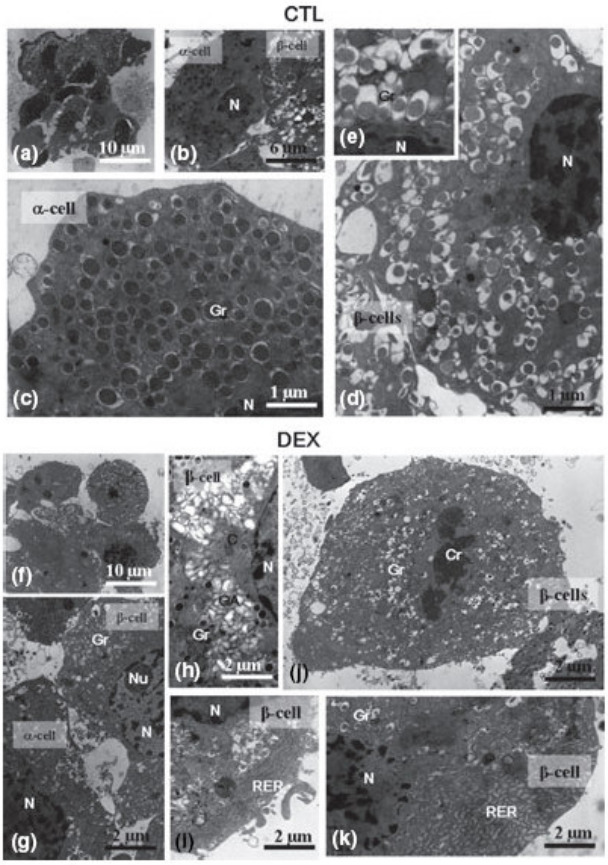

Figure 4 Transmission Electron Microscopy of isolated islets. General view of the islets (a, f); partial view of the cytoplasm of the alpha and beta cells showing the characteristic granules in CTL and DEX group (b, g, respectively); detailed view of the alpha-cell cytoplasm (c) and of the beta-cell cytoplasm in the CTL group (d) with details of granules (*inset*). Note the increase in the secreting organelles, such as the Golgi apparatus, in the beta-cell cytoplasm (h) and rough endoplasmic reticulum in the peripheral region (i, k) in DEX islet. Mitosis (initial telophasis) figure in the beta cell of DEX islet (j). N, nucleus; Gr, granules; C, centrioles; Cr, chromosomes; Nu, nucleolus; GA, Golgi apparatus; RER, rough endoplasmic reticulum.

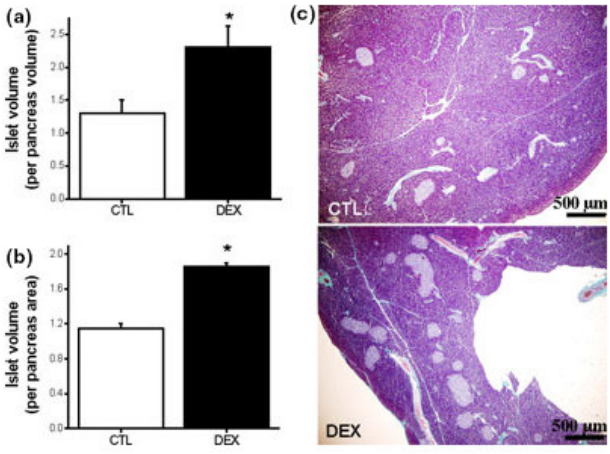

Figure 5 Increased pancreatic islet mass in insulin-resistant rats. Observe the significant increase in islet volume (a) and islet density (b) in DEX pancreas. Representative image of Gömori's trichrome-stained pancreas section from CTL and DEX groups (c). Data are mean ± SEM. *Significantly different *vs.* CTL. $P < 0.05$; $n = 5$. Unpaired Student's *t*-test. Magnification ×40.

Increased pancreatic islet area and mass in pancreas from DEX-treated rats

Morphometrical parameters indicate marked differences between DEX and CTL islets (Table 1). DEX treatment induced a significant increase in area and perimeter of the islets, compared with CTL islets ($n = 500$, $P < 0.05$). In addition, the roundness-factor value decreased in DEX islets ($n = 500$, $P < 0.05$). The increase in area and perimeter values was 2.1- and 1.5-fold, respectively, in DEX compared with CTL islets (Table 1). The pancreatic islet mass was 1.7-fold higher in DEX, compared with CTL pancreas ($n = 5$, $P < 0.05$; Figure 5a,c). Furthermore, the density of the islets was significantly higher in DEX compared with the CTL group ($n = 5$, $P < 0.05$; Figure 5b,c).

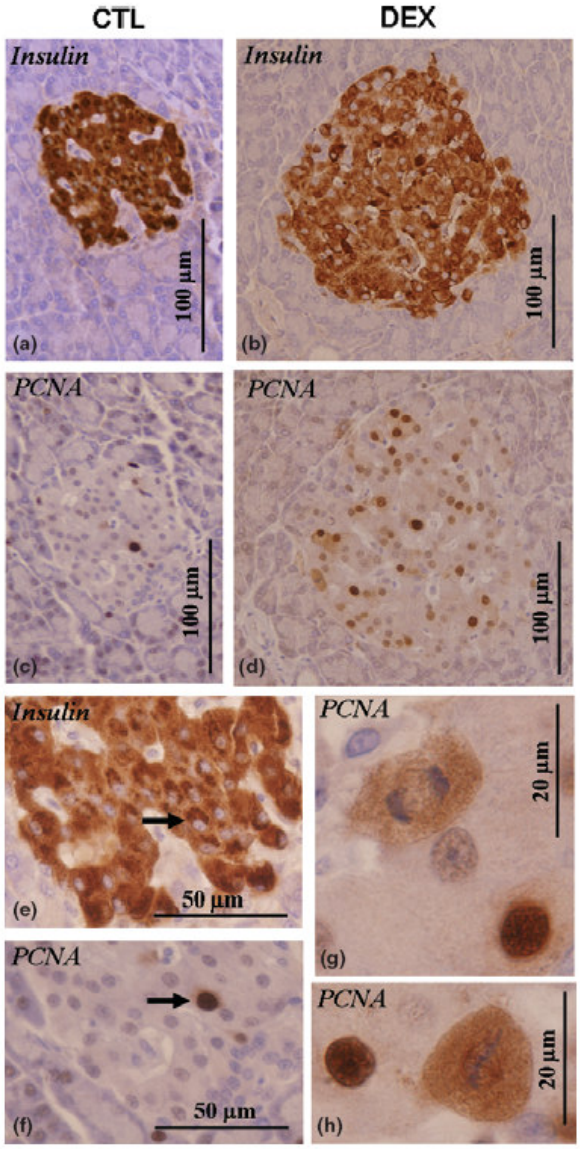

Figure 6 Light microscopy evaluation of *in situ* anti-PCNA expression in the isolated pancreatic islets. The majority of PCNA-positive cells were related to the beta cell (arrows). Two serial sections were made and one was submitted to anti-PCNA reaction (c, d) and the subsequent section was submitted to anti-insulin reaction (a, b). The increase in PCNA-proliferating cells was notable in DEX islets, including mitotic PCNA-positive figures (d, g, h).

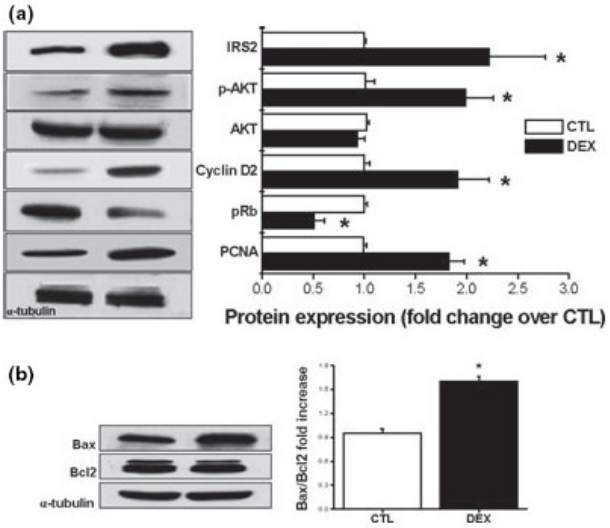

Figure 7 Increased expression of proteins related to the beta cell proliferation in insulin-resistant rats. Equal amounts of total protein were run in SDS-PAGE from CTL and DEX islets and detected with anti-IRS2, phosphorylated AKT, cyclin D_2, pRb and PCNA (a) antibodies. In (b) Bax/Bcl2 protein ratio. Data are mean ± SEM *Significantly different *vs*. CTL. $P < 0.05$; $n = 6$. Unpaired Student's *t*-test.

Immunohistochemistry of PCNA and quantification of beta-cell proliferation

The potential capacity of beta cells for proliferation in both CTL and DEX islets was evaluated at the immunohistochemical level. The PCNA-positive cell was observed in low frequency in pancreatic islets from CTL rats (Figure 6c). Serial section of pancreas stained for insulin confirms the co-localization of PCNA-positive nucleus with beta cell (Figure 6e,f). However, pancreatic beta cells in DEX islets exhibited marked increase in PCNA-positive nuclei (Figure 6d). Figure 6(g,h) shows two PCNA-positive beta cells undergoing mitosis in DEX islets. The quantitative determination of beta-cell proliferation by morphometric analysis revealed a significant increase in beta-cell proliferation in DEX islets ($n = 4$, 800 nuclei; $P < 0.05$). The percentage of PCNA-positive nuclei from total beta cells counted was (15.2 ± 1.2 *vs*. 2.37 ± 0.46% for DEX and CTL, respectively) (compare Figure 6c with Figure 6a and Figure 6d with Figure 6b).

Immunoblotting of islets for IRS-2, p-AKT, cyclin D_2, pRb, PCNA, Bax and Bcl-2 proteins

To determine whether the changes in pancreatic beta-cell proliferation in DEX islets were associated with alterations in proteins involved in this event, we performed immunoblotting for IRS-2, p-AKT, AKT, cyclin D_2, pRb and PCNA proteins. A 2.2-fold increase in IRS-2 protein expression was observed in DEX islets ($n = 6$, $P < 0.05$; Figure 7a). The phosphorylation levels (but not total protein content) of

AKT was also significantly increased in DEX compared with CTL islets ($n = 6$, $P < 0.05$; Figure 7a). Cyclin D_2 protein expression exhibited a 1.9-fold increase in DEX islets ($n = 6$, $P < 0.05$; Figure 7a), whereas the expression of total pRb was 0.5-fold decreased in DEX islets ($n = 6$, $P < 0.05$; Figure 7a). In addition, the expression of PCNA was markedly increased (1.8-fold) in DEX compared with CTL islets ($n = 6$, $P < 0.05$; Figure 7a). The ratio between pro-apoptotic Bax and anti-apoptotic Bcl2 proteins increased 1.6-fold in DEX islets ($n = 6$, $P < 0.05$; Figure 7b).

Discussion

Glucocorticoid-induced insulin resistance is a useful model for the study of the pathogenesis of insulin resistance. Recently, we demonstrated that short-term administration of different concentrations of DEX resulted in a varied degree of insulin resistance without overt installation of type 2 diabetes (Rafacho *et al*. 2008). The insulin-resistant rats used in the present study showed endocrine pancreas adaptations, as judged by the increase in islet function, mass and beta-cell proliferation. We showed, for the first time, in DEX-induced insulin resistance, that the potential mechanisms underlying these events are activation of the IRS-2/AKT pathway and cell cycle activation with the participation of cyclin D_2.

To compensate for insulin resistance, the endocrine pancreas undergoes morphological and functional alterations. We, herein, show that DEX rats exhibit hyperinsulinaemia and increased GSIS, supporting the functional improvement of islets in this condition. The increase in plasma insulin levels and the response to glucose of islets from glucocorticoid-treated rodents have been previously described by others (Ogawa *et al*. 1992; Barbera *et al*. 2001; Novelli *et al*. 1999; Rafacho *et al*. 2007).

Studies on the structure of pancreatic islets during insulin resistance induced by glucocorticoids have already been documented (Boquist 1972; Visser *et al*. 1979; Jonas *et al*. 1983; Tomita *et al*. 1984; Zwicker & Eyster 1993; Rafacho *et al*. 2007). The morphological alterations described by these authors included hypertrophy and hyperplasia of islets and beta cells, increase in islet vascularization and occasional mitotic figures. No alterations are observed with regard to the distribution of exocrine and endocrine cells. These results correlate well with hyperinsulinaemia (Boquist 1972; Visser *et al*. 1979; Tomita *et al*. 1984; Rafacho *et al*. 2007). Here, we have also observed several hypertrophic DEX islets with a reduction in roundness-factor and signs of hyperplasia of varied degrees. Increased vascularization was also observed (data not shown); furthermore, the normal distribution of insulin-positive cells within the islets agrees with previous studies in hypertrophic islets (Spencer *et al*. 1986; Zwicker & Eyster 1993). However, glucagon-positive cells exhibited an apparent discontinued ring in DEX islets. In accordance with this observation, islets from KKAy mice show a reduction in alpha-cell area and these cells projected from the periphery to the centre; this projection was often sparse (Diani *et al*. 1987). Nevertheless, the absolute number of alpha cells is not altered in these mice islets; instead, the relative area reduction reflects the increased absolute beta-cell number, which is in agreement with our results for beta-cell proliferation in DEX islets.

Ultrastructural studies of islets during endocrine pancreatic dysfunction are scarce (Jonas *et al*. 1983; Janssen *et al*. 2003; Momose *et al*. 2006). These studies show modifications in the relative amount and aspect of some organelles, including secretory granules. In the present study, we observed an increase in secreting organelles, such as endoplasmic reticulum and Golgi apparatus in DEX islets, indicating hyperfunction. Similar alterations in secreting organelles occur in a diabetic model, the 21-week-old GK mice, at a few weeks before the development of overt diabetes. The beta cells of these mice present cytoplasm occupied with large amounts of enlarged small-sized endoplasmic reticulum with a reduction in the number of insulin granules (Momose *et al*. 2006).

The relative and absolute endocrine cell area or volume, especially of the beta cell, can be modified by experimental and pathophysiological conditions (Tomita *et al*. 1984; Zwicker & Eyster 1993; Gómez Dumm *et al*. 1995; Janssen *et al*. 2001). Morphometric analysis of some quantitative parameters was then performed to evaluate possible changes during the onset of insulin resistance. An increase in islet area and perimeter, as well as in the absolute mass and relative density, of the islets in DEX pancreas was observed. These results are in accordance with previous observations and indicate an adaptive mechanism to compensate for peripheral insulin action impairment imposed by DEX (Tomita *et al*. 1984; Ogawa *et al*. 1992; Rafacho *et al*. 2007). Another revealing result obtained in this study is the marked increase in PCNA staining in beta cells from DEX islets. DEX islets exhibited an increase of more than sixfold in beta-cell proliferation. PCNA is an indicator for cell proliferation and has been used to determine beta-cell mass expansion (Zhang *et al*. 2005; Lipsett *et al*. 2006; Terauchi *et al*. 2007; Vasavada *et al*. 2007). The marked increase in the ratio of beta-cell proliferation in DEX islets was not accompanied by a similar increase in islet mass. The maintenance of beta-cell mass is a dynamic process resulting from a balance between neogenesis, proliferation, cell volume changes

and cell death (Bonner-Weir 2000, 2001). It was demonstrated recently that the absolute increase in beta-cell proliferation might not reflect an increase in beta-cell mass in a glucose-infused mouse model (Alonso *et al.* 2007). Herein, the 6.4-fold increase in beta-cell proliferation includes all cells that have been through S phase during the 5-day administration of DEX. However, we observed an increase in Bax/Bcl2 protein ratio indicating a positive trend for activation of cell death in DEX rats. Thus, these data could explain, in part, the moderate increase in absolute islet mass in DEX rats.

Literature reporting the control of cell cycle events in beta cells is recent, in part, because of a long-standing belief that beta cells cannot replicate. Several groups have tried to elucidate the control of the beta-cell mass (see a list of works in recent reviews: Heit *et al.* 2006; Vasavada *et al.* 2006). Herein, we found a marked increase in islet mass and beta-cell proliferation, accompanied by an increase in IRS-2 and p-AKT levels in insulin-resistant animals. Several approaches, *in vivo* or *in vitro*, with pancreatic beta cells have confirmed the participation of growth factors in the modulation of beta-cell proliferation and expansion (Villanueva-Peñacarrillo *et al.* 1999; Garcia-Ocaña *et al.* 2000; Amaral *et al.* 2004; Friedrichsen *et al.* 2006; Okada *et al.* 2007). Between the several pathways most known to mediate beta-cell proliferation is the IRS-2/PI3K/AKT/p70S6K cascade (Lingohr *et al.* 2002; Okada *et al.* 2007). We believe that high circulating insulin, rather than mild glucose levels, exerts a positive and autocrine effect on beta-cell proliferation via insulin signalling in DEX-treated rats.

Consistent with this result, insulin infusion stimulates beta-cell proliferation and increases beta-cell mass in rats (Paris *et al.* 2003). Moreover, a recent study showed that insulin, but not IGF-1, is crucial to the glucose-stimulated beta-cell proliferation. Incubating MIN6 cells with high glucose in the presence of an anti-insulin antibody almost abolished the rate of proliferation (Muller *et al.* 2006). Studies in mice lacking IRS-2 further support the role of insulin in beta-cell replication (Withers *et al.* 1998; Kushner *et al.* 2002). Taken together, these data implicate the IR/IRS-2/PI3K/AKT pathway as a mediator of beta-cell proliferation in insulin-resistant rats.

The increase in cyclin D_2 and decrease in pRb protein content were also observed in DEX islets. Activation of AKT in pancreatic beta cells resulted in a marked expansion of beta-cell mass, due to an increase in beta-cell proliferation and size, which is associated with increased cyclin D_1, cyclin D_2, and p21 levels and CDK4 activity (Fatrai *et al.* 2006). Thus, in DEX rats, the increased levels of phosphorylated AKT might maintain, at least partially, the higher levels of cyclin D_2 protein. Recently, pRb loss in pancreatic islets was observed not to result in increased islet volume, density and beta-cell proliferation (Vasavada *et al.* 2007). Although pRb seems not to be essential in the transgenic mice model, we cannot rule out the possible participation of diminished pRb protein content, favouring the release of cell cycle progression in our DEX rats. Finally, we did not address whether the alterations in the protein contents are mediated by a direct effect of DEX. However, two lines of evidence favour the idea that this alteration is the result of indirect insulin resistance rather than a direct effect of DEX treatment. First, DEX incubation of airway smooth muscle cells induced cell proliferation inhibition, concomitant with decreased cyclin D_1 protein expression and decreased phosphorylation of pRb protein. Secondly, in transgenic mice models ($IR^{+/-}$, IRS-$1^{-/-}$, $IR^{+/-}$/IRS-$1^{+/-}$) that exhibit insulin resistance and hyperinsulinaemia, the IRS-2 pathway drives beta-cell hyperplasia (Araki *et al.* 1994; Tamemoto *et al.* 1994; Bruning *et al.* 1997; Kido *et al.* 2000). Zucker fatty rats (Pick *et al.* 1998) also present hyperinsulinaemia, mild blood glucose levels and beta-cell hyperplasia like DEX rats. Based on these data, we are tempted to believe that the proteins altered herein (IRS-2, AKT and cyclin D2), which are potential mediators of beta-cell hyperplasia, are positively regulated by circulating insulin.

In summary, we have generated an insulin-resistant animal by short-term administration of DEX. Under these conditions, compensations by the beta cell are observed, including increases in islet function, islet mass and beta-cell proliferation. The potential mechanisms that underlie these events are the activation of the IRS-2/AKT pathway and cell cycle activation, mediated by cyclin D_2.

Acknowledgements

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ARTIGO 3

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High Doses of Dexamethasone Induce Increased Beta-cell Proliferation in Pancreatic Rat Islets

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Running head: Beta-cell proliferation in DEX rats

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ABSTRACT

Activation of insulin signaling and cell cycle intermediates are required for adult beta-cell proliferation. Here, we report a model to study beta-cell proliferation in living rats by administering three different doses of dexamethasone (0.1, 0.5 and 1.0 mg/kg, *ip.*) (DEX 0.1, DEX 0.5 and DEX 1.0) for 5 days. Insulin sensitivity, insulin secretion and histomorphometric data were investigated. Western blotting was used to analyze the levels of proteins related to the control of beta-cell growth. DEX 1.0 rats, which present moderate hyperglycemia and marked hyperinsulinemia, exhibited a 5.1-fold increase in beta-cell proliferation and an increase (17%) in beta-cell size, with significant increase in beta-cell mass, compared to control (CTL) rats. The hyperinsulinemic but euglycemic DEX 0.5 rats also showed a significant 3.6-fold increase in beta-cell proliferation. However, DEX 0.1 rats, which exhibited the lowest degree of insulin resistance, compensate for insulin demand by improving only islet function. Activation of the IRS2/PI3K/AKT/p70S6K pathway, as well as pRb in islets from DEX 1.0 and DEX 0.5, but not in DEX 0.1, rats was also observed. Therefore, increasing doses of dexamethasone induce three different degrees of insulin requirement in living rats, serving as a model to investigate compensatory beta-cell alterations. Augmented beta-cell mass involves beta-cell hyperplasia and, to a lower extent, beta-cell hypertrophy. We suggest that alterations in circulating insulin and, to a lesser extend, glucose levels could be the major stimuli for beta-cell proliferation in the dexamethasone-induced insulin resistance.

Keywords: beta-cell growth – glucocorticoid – hyperglycemia – hyperinsulinemia – insulin resistance

INTRODUCTION

Pancreatic beta cells are the only significant source of insulin, which is required for maintaining appropriate metabolic homeostasis and particularly to maintain glucose levels within a narrow range. However, failure of the beta-cell capacity (beta-cell dysfunction and/or insufficient beta-cell mass) contributes to the pathogenesis of both type 1 (T1DM) and type 2 *diabetes mellitus* (T2DM) (10, 18). Physiological or pathological states such as aging, pregnancy, insulin resistance and obesity demand an increase in circulating insulin. Several beta-cell adaptations are observed in these conditions, including increased insulin synthesis and secretion, hyperplasia and hypertrophy (23, 28, 30). Beta-cell mass plays an essential role in limiting the amount of insulin that is secreted in these systemic conditions of increased insulin demand. Patients with T2DM show reduced beta-cell mass as a result of impaired beta-cell proliferation and/or increased beta-cell apoptosis, suggesting that adequate beta-cell mass is required for prevention of diabetes (10, 18). Although remarkable improvements in the management of diabetic patients have occurred over recent years, new therapies are still needed to further improve metabolic control of this pathology (36).

Amelioration of beta-cell function and increase in beta-cell number are important goals in diabetes research. Pancreatic beta-cell mass results from a dynamic balance between cell growth (neogenesis, proliferation, and size) and beta-cell death (apoptosis) (7). It has been proposed that most adult mouse beta cells are derived from mitosis of preexisting differentiated beta cells rather than neogenesis from stem cells (13). The tyrosine kinase, lactogen, incretin and growth factor pathways have all been demonstrated to have an important role in the control of beta-cell growth. With particular attention, the insulin signaling pathway (through insulin receptor substrate 2 (IRS2) signaling intermediate), and

the cyclin D_1 and D_2, cyclin-dependent-kinase 4 (CDK4), and protein retinoblastoma (pRb) proteins have been shown to largely mediate adult beta-cell growth (reviewed in 1, 15, 41). The majority of reports on the control of beta-cell growth have been carried out using genetic mice models; these models characterize well the mechanisms of beta-cell differentiation and function during embryogenesis, and postnatal periods. Another interesting approach to investigate beta-cell growth is the use of adult rodent models, such as the high-fat diet-induced obesity, pregnancy and pancreatectomy models (10, 11, 27). There is no doubt that these genetic and adult models have furthered the understanding of beta-cell differentiation and function. However, intrinsic adaptive mechanisms in genetic models or the appearance of circulating factors in adult models could mask the expected physiological beta-cell compensation in these models. Recently, a short-term glucose infusion model was developed to directly stimulate beta-cell proliferation and mass in mouse and rat pancreas (2, 16). However, the mouse model did not exhibit increases in beta-cell mass, size and islet number (2) and beta-cell proliferation was not found increased in the rat model (16).

We have recently demonstrated that the administration of different doses of dexamethasone for 5 days in adult rats induces hyperinsulinemia and insulin resistance in a dose-dependent fashion without the appearance of marked hyperglycemia (31). The decreased action of insulin peripherally induces compensatory adaptations in the endocrine pancreas (44). In order to confirm whether this hypothesis affects beta-cell growth and death, we evaluated the beta-cell proliferation, size and death and the levels of the important proteins involved in insulin signaling and in the control of beta-cell cycle in three different degrees of insulin requirement in living rats.

MATERIALS AND METHODS

Materials. Dexamethasone phosphate (Decadron®) was from Aché (Campinas, SP, Brazil). Sodium thiopental (THIOPENTAX®) was from Cristália (Itapira, SP, Brazil). Human recombinant insulin (Biohulin® N) was from Biobrás (Montes Claros, MG, Brazil). The reagents used in the insulin secretion protocol and RIA were from Mallinckrodt Baker, Inc. (Paris, Kentucky, France) and from Sigma (St. Louis, MO, USA). The ^{125}I-labeled insulin (human recombinant) for RIA assay was purchased from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK). SDS-PAGE and immunoblotting were performed using Bio-Rad systems (Hercules, CA, USA) and all chemicals used were from Bio-Rad (Hercules, CA, USA) and from Sigma (St. Louis, MO, USA). Anti insulin (rabbit polyclonal), anti serine-threonine kinase (AKT) (rabbit polyclonal), anti phosphorylated AKT (Ser473) (pAKT) (rabbit polyclonal), anti ribosomal protein S6 kinase (p70S6K) (mouse monoclonal), anti p21 (mouse monoclonal), anti Bcl2 (rabbit polyclonal), anti phosphorylated extracellular signal-regulated kinase 1/2 (pERK) (Tyr204) (mouse monoclonal) and anti α-tubulin (mouse monoclonal) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti proliferating cell nuclear antigen (PCNA) (mouse monoclonal) was from DakoCytomation (Carpinteria, CA, USA). Anti IRS2 (rabbit polyclonal) was from Cell Signaling Technology (Beverly, MA, USA). Anti p-85 regulatory subunit of phosphatidylinositol 3-kinase (PI3K) (mouse monoclonal) and anti extracellular signal-regulated kinase 1/2 (ERK) (rabbit polyclonal) was from Upstate (Lake Placid, NY, USA). Anti pancreatic-duodenal homeobox 1 (PDX1) (rabbit polyclonal) was from Chemicon (Temecula, CA, USA). Anti cyclin D_1 (mouse monoclonal) and anti-cyclin D_2 (mouse monoclonal) were from Lab Vision Corporation (Fremont, CA, USA). Anti cyclin-dependent-kinase 4 (CDK4) (mouse monoclonal) was from Abcam (Cambridge, MA, USA). Anti phospho-pRb (mouse

monoclonal) and total pRb (mouse monoclonal) was from BD Biosciences (Mississauga, ON, CA).

Animals. Experiments were performed on groups of male Wistar rats (3-months old) obtained from the State University of Campinas Animal Breeding Center and kept at 24ºC on a 12 h light/dark cycle. The rats had access to food and water *ad lib*. The experiments with animals were approved by the institutional State University of Campinas Committee for Ethics in Animal Experimentation and conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23 revised 1996).

Dexamethasone treatment. The rats received daily i.p. injection, at a time between 7:30 – 8:30 h, for 5 consecutive days. Rats were divided into four groups, according to the amount of drug administered (mg/kg body weight, dissolved in saline): 0.1 mg/kg dexamethasone (DEX 0.1), 0.5 mg/kg dexamethasone (DEX 0.5), 1.0 mg/kg dexamethasone (DEX 1.0) and saline alone (CTL). All dexamethasone-treated (DEX-treated) rats exhibit a significant dose-dependent suppression of endogenous corticosterone levels at the end of dexamethasone-treatment. Additionally, it is observed a positive correlation with the adrenal gland mass and serum corticosterone levels (31).

Blood glucose and serum insulin content. On the day following the last DEX or saline administration, blood was collected (≅ 8:00 am) from the tail of fed rats to measure blood glucose levels with a glucometer ("one touch" - Johnson & Johnson). After the sacrifice

(exposure to CO_2 followed by decapitation) the trunk blood was collected. The serum, obtained by centrifugation, was used to measure the insulin content by RIA, utilizing a Guinea-pig anti-rat insulin antibody and rat insulin as standard (35).

Intraperitoneal insulin tolerance test (ipITT) and Insulin content and secretion. These procedures were performed in separates groups of fed rats (≅ 8:00 am) as described in detail, previously (31, 32).

Quantitative approaches in endocrine pancreas. To study the morphometric parameters of endocrine pancreas, 8 pancreases from each group were excised and processed according to a previous description (33).

Immunostaining: Cellular distributions of insulin, PCNA and PDX1 were analyzed according to a previous description (33). *Beta-cell mass:* Beta-cell mass was determined by point counting morphometry on each pancreas section immunostained for insulin according previous descriptions (30, 33, 43). Each section was systematically scored with a grid of 100 points (final magnification ×320). The numbers of intercepts over beta-cells, endocrine non-beta-cells, exocrine pancreatic tissue and non-exocrine pancreatic tissue were counted. The beta-cell relative volume was calculated by dividing the intercepts over beta-cells by the intercepts over the total pancreatic tissue; the beta-cell mass was then estimated by multiplying the beta-cell relative volume by the total pancreas weight. A minimum of 500 fields per pancreas were counted. *Beta-cell size:* Average beta-cell size was measured using the same material used for the determination of beta-cell mass. Total beta-cell area inside the islet was measured with KS-300 quantitative software (Carl Zeiss Jena GmbH, Jena, Germany) and at least 80 islets/group (5,200 ± 200 beta-cell nuclei per group) were sampled.

The average cell size was determined by dividing the measured total beta-cell area by the number of beta-cell nuclei within each total beta-cell area sampled, according to a previous description (6, 28, 43). *Beta-cell proliferation:* Averaged beta-cell proliferation was obtained by counting total islet-cell nuclei stained for insulin and PCNA using the same software cited above. At least 40 islets/group (3,437 ± 435 beta-cell nuclei) were sampled. The beta-cell proliferation was estimated by the percentage of PCNA-positive cells from the total of insulin-positive cells (20, 39, 42). *Beta-cell death:* Detection of DNA fragmentation in situ was visualized with the use of the ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (Chemicon International, Serologicals Corporation, Norcross, GA, USA). Staining was evaluated using a light microscope after counterstaining with methyl green. Averaged beta-cell death was obtained by counting total islet-cell nuclei stained for insulin and TUNEL (In situ DNA end labelling method) using the same software cited above. At least 40 islets/group (3,120 ± 270 beta-cell nuclei) were sampled. The beta-cell death was estimated by the percentage of TUNEL-positive cells from the total of insulin-positive cells (24). All the images for documentation were registered by a Sony CCD-IRIS-RGB camera, coupled to a Zeiss Axioskop 2 microscope (Carl Zeiss GmbH, Eching bei München, Germany). *Islet area:* Islet area were determined as previously described in detail (32, 33).

Protein extraction and immunoblotting. Protein extraction and immunoblotting were carried out as previously reported (33). Pools of isolated islets were homogenized in ice-cold cell lysis buffer (Cell Signaling, MA, USA). Protein concentration from total cell lysate was determined by the RCDC method, according to the manufacturer (Bio-Rad, CA, USA). Protein obtained from islets (100 µg) was used for each experiment. Immunoblotting experiments were performed at least six times using different samples (each sample consisting of islets obtained

from one rat). After 2 h of blocking at RT, membranes containing islet lysates were washed in TBST and incubated overnight with the appropriate primary antibodies. After washing in TBST, membranes were incubated with the appropriate secondary antibody. Antibody binding was detected by enhanced SuperSignal® West Pico Chemiluminescent Substrate (PIERCE, Rockford, IL, USA), as described by the manufacturer.

Statistical Analysis. Results are expressed as the means ± S.E.M. of the indicated number (*n*) of experiments. Analysis of variance (one way - ANOVA) for unpaired groups, followed by Tukey *post test* was utilized for multiple comparisons of parametric data. The significance level adopted was $P < 0.05$.

RESULTS

Insulin resistance induced by dexamethasone. We have recently characterized the metabolic status and the insulin secretion of pancreatic islets in three models of DEX-induced insulin resistance (31). We, herein, confirm that 5-day DEX administration at different concentrations reproduces dose-dependent peripheral insulin resistance in rats, as judged by the ipITT. The *K*itt values were 1.83 ± 0.16, 1.38 ± 0.22, 0.81 ± 0.11 and 0.47 ± 0.07 %/min in CTL, DEX 0.1, DEX 0.5 and DEX 1.0, respectively ($n = 6$, $P < 0.01$ for DEX 0.5 and DEX 1.0 *vs* CTL rats; Fig.1A). The increase in serum insulin values also exhibited a dose-dependent pattern of 4.7 ± 0.4, 14.2 ± 1.9, 31 ± 4.4 and 44 ± 8 ng/ml for CTL, DEX 0.1, DEX 0.5 and DEX 1.0, respectively ($n = 10$, $P < 0.01$ for all DEX groups *vs* CTL rats; Fig. 1B). The augmented levels of serum insulin were sufficient to prevent hyperglycemia in DEX 0.1 and DEX 0.5 rats,

however, not enough to avoid moderate hyperglycemia in DEX 1.0 rats (115 ± 2.3, 113 ± 3.6, 109 ± 4.0 and 175 ± 29.7 mg/dl for CTL, DEX 0.1, DEX 0.5 and DEX 1.0, respectively; n = 10, $P < 0.05$ for DEX 1.0 *vs* CTL, DEX 0.1 and DEX 0.5 rats; Fig 1C). Finally, glucose-stimulated insulin release (GSIR) was 3.8 ± 0.2, 9.7 ± 0.5, 12.8 ± 0.8 and 13.2 ± 1.0 % of islet content in CTL, DEX 0.1, DEX 0.5 and DEX 1.0, respectively (n = 12, $P < 0.001$ for all DEX groups *vs* CTL rats; Fig. 1D).

Islet size and beta-cell mass in insulin-resistant rats. Mean islet area enlarged by 1.2-fold in DEX 0.5 and by 1.7-fold in DEX 1.0 islets ($P < 0.001$ for DEX 0.5 and DEX 1.0 *vs* CTL islets). The islet area was 15,655 ± 324, 16,611 ± 327, 18,957 ± 376 and 26,794 ± 526μm^2 for CTL, DEX 0.1, DEX 0.5 and DEX 1.0, respectively ($n \cong 730$ islets per group). Sections stained for insulin exhibited marked differences in size and number of islets in DEX 0.5 and DEX 1.0 compared to CTL pancreas (Fig. 2A). The total pancreas weight was significantly reduced in all DEX-treated compared to CTL rats (n = 5, $P < 0.05$; Fig. 2B). Morphometric analysis revealed that the number of islets per pancreatic area augmented significantly in DEX 0.5 and DEX 1.0 (30% and 31% *vs* CTL, respectively, $P < 0.01$) (Fig. 2C) and that the relative beta-cell mass was significantly enhanced in DEX 0.5 and DEX 1.0 rats (2.3- and 2.7-fold *vs* CTL, $P < 0.05$, respectively) (Fig. 2D). Differences in the absolute beta-cell mass were also observed in DEX 1.0 compared to CTL rats (1.0 ± 0.08, 1.34 ± 0.19, 1.69 ± 0.23 and 1.86 ± 0.20 mg for CTL, DEX 0.1, DEX 0.5 and DEX 1.0, respectively; $P < 0.05$, n = 8; Fig. 2E, Table 1).

Enlargement of beta-cell size and increases in beta-cell proliferation in insulin-resistant rats. Figure 3A shows a large distribution of PCNA-positive nuclei in DEX 0.5 and DEX 1.0,

compared with CTL islets; the majority of these PCNA-positive nuclei were co-localized with insulin-positive cells (arrows in Fig. 3A). We observed significant increases in individual beta-cell size of 9% and 17 % in DEX 0.5 and DEX 1.0, respectively, *vs* CTL group ($n \cong$ 5,200 nuclei, $P < 0.01$) (Fig. 3B and Table 1). In addition, the beta-cell proliferation expanded markedly, reaching 3.6- and 5.1-fold in DEX 0.5 and DEX 1.0 rats, respectively ($P < 0.001$ *vs* CTL) (Fig. 3C and Table 1); values were 2.8 ± 0.4, 3.1 ± 0.3, 10.3 ± 0.6 and 14.5 ± 0.9 % of total insulin-positive cells for CTL, DEX 0.1, DEX 0.5 and DEX 1.0, respectively ($n \cong$ 3,500 nuclei).

Beta-cell death is unchanged in insulin-resistant rats. We stained pancreas sections with insulin or with TUNEL to detect fragmented DNA. Islets from CTL and all DEX-treated rats showed very low levels of beta-cell death, which were co-localized with insulin-positive cells (data not shown). Quantification of TUNEL revealed no difference in beta-cell death frequency among the groups (Table 1, 2). The levels of anti-apoptotic protein Bcl2 were not altered in the islet lysates of any of the four experimental groups (data not shown).

Insulin signaling and cell cycle components in insulin-resistant rats. Protein content of IRS2 in islet lysates was significantly increased in DEX 1.0 rats (2.2-fold vs. CTL; $P < 0.05$; Fig. 4A). PI3K protein levels (p85 regulatory subunit) also increased significantly, and were 40% higher in DEX 1.0 than CTL islets ($P < 0.05$). Islet lysates from all groups did not exhibit differences in AKT protein expression. However, Western blotting, using an anti-phospho AKT (Ser473) antibody revealed that DEX 0.5 and DEX 1.0 islets exhibited a significant increase in the level of AKT protein phosphorylation (1.85- and 1.98-fold *vs* CTL, respectively, $P < 0.05$; Fig. 4A). A marginal increase in the AKT phosphorylation was also

observed in DEX 0.1 islets (1.44-fold *vs* CTL; NS). The p70S6K protein expression was enhanced (29%) in DEX 1.0 compared with CTL islets ($P < 0.05$; Fig. 4A). PDX1 protein content was increased by 1.44- and 1.59-fold in DEX 0.5 and DEX 1.0 islet lysates compared with CTL, respectively ($P < 0.05$; Fig. 4A). Qualitative analysis of PDX1 in pancreas sections confirmed the large distribution of this protein on pancreatic beta-cell nuclei in all groups (Fig. 4B). In all groups, the reaction was also positive for the cytoplasm of beta-cells, since PDX1 is translated in this compartment.

Islet levels of cyclin D_1 protein were not altered among the four groups (data not shown). However, cyclin D_2 protein significantly increased in DEX 0.5 and DEX 1.0 by 1.51- and 1.91-fold compared to CTL, respectively ($P < 0.01$ only for DEX 1.0; Fig. 5). DEX 0.5 and DEX 1.0 rats showed higher levels of CDK4 protein compared to CTL rats (39% and 43%, respectively; $P < 0.05$; Fig. 5). The total pRb protein content was reduced by 49% in DEX 1.0 islet lysates, compared to CTL islets ($P < 0.05$; Fig. 5). A significant increase in p-pRb protein levels was observed in DEX 1.0 islets (2.51-fold *vs* CTL; $P < 0.05$; Fig. 5). Although not significantly different, a tendency of p-pRb to increase was noticed in DEX 0.1 and DEX 0.5, compared with CTL islets. The levels of p21 and phosphorylated ERK proteins tended to increase in DEX 0.5 and DEX 1.0 islet lysates, but did not reach statistical significance when compared with CTL (data not shown). The protein content of ERK was similar in islet lysates from all groups (data not shown). Finally, confirming the immunostaining for PCNA, the PCNA protein levels were higher in DEX 0.5 and DEX 1.0, compared with CTL islets ($P < 0.01$; Fig. 5). These data are summarized in Table 1.

DISCUSSION

At present, we generate three different degrees of insulin resistance through administration of increasing concentrations of dexamethasone (summarized in Table 1). Rats exhibiting marked insulin resistance, together with marked hyperinsulinemia and moderate hyperglycemia (DEX 1.0), presented a remarkable increase in beta-cell proliferation and a modest increase in beta-cell size, with a significant increase in beta-cell mass. The rats with moderate insulin resistance, concomitant with high circulating insulin and normal blood glucose levels (DEX 0.5), exhibited increased beta-cell proliferation and size. However, rats that exhibited only slight insulin resistance (DEX 0.1), and were normoglycemic and hyperinsulinemic, did not show alterations in beta-cell proliferation and size and presented a pancreas morphology that was quite similar to that of the CTL rats. These results suggest a role for glucose and, especially for insulin, as mitogenic signals to induce beta-cell proliferation *in vivo*. Additionally, in DEX 1.0, and to a lesser extent in DEX 0.5 rats, the IRS2/PI3K/AKT/p70S6K pathway and PDX1 were up-regulated, indicating a positive involvement of the PI3K cascade in such an effect in adult rats. In the same manner, increases in cyclin D_2 and CDK4 protein levels and the phosphorylated state of pRb in DEX 1.0 islets indicate the participation of these proteins in the postnatal control of beta-cell expansion during insulin resistance induced by dexamethasone.

Glucose has been suggested as a stimulus for beta-cell proliferation (6, 8, 26, 37, 40). Short-term infusion of 50% glucose in mice constitutes a model of moderate hyperglycemia and hyperinsulinemia. These mice show increases in beta-cell proliferation without alterations in beta-cell size and mass (2). Furthermore, it has been recently demonstrated that glucose metabolism is required for compensatory beta-cell proliferation during insulin resistance induced by high-fat diet (39). Based in these observations, we can suggest glucose as an

important stimulus for beta-cell proliferation, at least in DEX 1.0 rats. These rats presented the highest increase in beta-cell proliferation among the 4 groups. The major metabolic difference between DEX 1.0 and the other DEX groups is that the former exhibits moderate hyperglycemia (similar to that of mice receiving 50% glucose infusion). However, DEX 1.0 rats had a 9-fold increase in circulating insulin, compared to CTL rats, whereas mice receiving the 50% glucose infusion had a 2.25-fold increase in circulating insulin compared to matched controls (2). Several studies have reported an effect of the glucose-independent stimulus on beta-cell proliferation, probably because the glucose effect is mediated by insulin (22, 24, 25). The decrease in caspase-9 activity and increase in BrdU incorporation, induced by glucose, were blocked by the addition of anti-insulin antibody or by an inhibitor of PI3K in MIN6 cells (22). Mice that were knocked out for the insulin receptor, but not those knocked out for insulin-like growth factor 1 receptor in pancreatic beta cells fail to manifest compensatory islet hyperplasia in response to high-fat diet-induced insulin resistance (25). These observations point to insulin as a crucial signal for mediating this process and are in agreement with the euglycemic DEX 0.5 rats, that exhibit 6.5-fold increase in serum insulin values, and marked increase in beta-proliferation. Nevertheless, we cannot discount a positive role for blood glucose in beta-cell proliferation for DEX 0.5 rats. Considering that these animals had moderate insulin insensitivity, this may favor the development of transiently hyperglycemia in certain periods (i.e., during absorptive periods). DEX 0.1 rats, although hyperinsulinemic (3-fold *vs* CTL rats), did not exhibit alterations in beta-cell growth. These rats developed only a slight degree of insulin resistance and their demand for insulin was compensated by an increase in islet function, sufficient to maintain glucose at normal values, as observed previously (31).

In adults, the beta-cell mass is maintained via a balance of beta-cell proliferation, size, neogenesis, and beta-cell death (7). Autopsy studies in humans (10) and numerous rodent models (2, 16, 25, 28, 33, 39) have shown that the insulin-resistant state disrupts this regulation and alters beta-cell growth and survival, promoting a compensatory increase in beta-cell mass, which corroborate our results with beta-cell mass in DEX 1.0 rats. The average values of beta-cell mass in DEX 0.5 and DEX 1.0 pancreas were 69% and 86% higher in relation to the control, respectively, justifying the increase of 3.6 and 5.1-fold in the proliferation rate and 9% and 17% in the beta-cell size, respectively. Thus, the tendency towards increased beta-cell mass in DEX 1.0 and DEX 0.5 rats seems to be a result of increased beta-cell hyperplasia and, to a lower extent, by beta-cell hypertrophy.

The insulin signaling pathway has been shown to largely mediate the adult beta-cell growth. IRS2 protein content was increased in DEX 1.0 islets. The importance of IRS2 in beta-cell expansion is well known (11, 27, 39, 45). Overexpression of IRS2 in pancreatic beta cells of glucokinase$^{+/-}$ mice feeding on a high-fat diet led to an improvement in glucose tolerance by increasing beta-cell mass, rather than function (39). AKT has been also implicated in the regulation of cell growth (16). Overexpression of AKT in mice leads to a marked increase in beta-cell mass and proliferation (5). Moreover, AKT can favor beta-cell expansion by inhibiting apoptosis and inducing cell cycle arrest by increasing p21 content (21). Our present results demonstrating AKT phosphorylation in DEX 1.0 and DEX 0.5 rats corroborate the positive modulation attributed to the PI3K/AKT effect on the beta-cell expansion. Similarly, p70S6K, an effector of the PI3K/AKT pathway, has also been demonstrated to exert a positive effect on beta-cell function and growth (41), supporting our observation of an increased p70S6K protein level in DEX 1.0 islets. Taken together, these data reinforce the role of insulin signaling in beta-cell proliferation by activating the IRS2/PI3K/AKT/p70S6K pathway, as

observed in islets from DEX 0.5 and DEX 1.0 rats.

Consistent with the activation of insulin signaling, we also observed an increase in cyclin D_2 and CDK4 content and in the phosphorylation status of pRb in DEX 1.0 islets. DEX 0.5 rats also revealed an increase in CDK4 and, although not significant, a tendency towards increased cyclin D_2 and phosphorylated pRb. The importance of cyclins D_1, D_2, CDK4 and p21 for adequate beta-cell proliferation has been demonstrated (12, 19, 46). No alterations in cyclin D_1 content and in status phosphorylation of ERK protein were noticed in DEX 0.5 and DEX 1.0 islets. However, augmented cyclin D_2 and CDK4 were observed in these rats, suggesting a pivotal role for cyclinD_2/CDK4 in the post-natal control of beta-cell expansion in insulin resistance.

Whether the alterations observed in endocrine pancreas in all DEX-treated rats resulted from direct dexamethasone action was not addressed herein. However, some data favor the idea that they resulted from indirect insulin resistance. Firstly, human cultured airway smooth muscle cells, in the presence of dexamethasone, show cell proliferation inhibition, concomitant with decreased cyclin D_1 protein expression and pRb phosphorylation (14). Secondly, *in vitro* experiments showed that dexamethasone induces cell death in insulin-secreting INS-1 cells (4, 34). Thirdly, in transgenic mice models ($IR^{+/-}$, $IRS1^{-/-}$, $IR^{+/-}/IRS1^{+/-}$) that developed insulin resistance and hyperinsulinemia, the IRS2 pathway drives beta-cell hyperplasia (3, 9, 17, 38). These observations support the idea that *in vivo* pancreas alterations do not result from a direct effect of dexamethasone administration.

In conclusion, using different doses of dexamethasone, we demonstrated that beta-cell proliferation and mass are much higher in rats that exhibit a marked hyperinsulinemia together with moderated hyperglycemia. This phenomenon seems to be dependent on the activation of the IRS2/AKT/PI3K/p70S6K pathway, as well as cyclin D_2, CDK4 and pRb. We suggest that

an increase in circulating insulin together with a moderate increase in blood glucose levels could be the major stimuli for beta-cell proliferation in the dexamethasone-induced insulin resistance.

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TABLES

Table 1 Summary of functional, structural and transductional adaptation in control (CTL) and dexamethasone-treated rats (DEX 0.1, DEX 0.5 and DEX 1.0).

	CTL	DEX 0.1	DEX 0.5	DEX 1.0
insulin resistance	absent	slight	moderate	marked
	functional adaptation			
↑ insulinemia	-	+	++	++
↑ GSIS	-	+	++	++
	structural adatation			
↑ beta-cell mass	-	-	-	+
↑ beta-cell size	-	-	+ +	+ + +
↑ beta-cell proliferation	-	-	+ +	+ + +
↑ beta-cell death	-	-	-	-
	transductional adaptation			
↑ IRS2/PI3K/AKT pathway	-	-	+	+ +
↑ p-ERK, ERK	-	-	-	-
↑ PDX1	-	-	+	+
↑ cyclyn D_2,CDK4	-	-	+	+ +
↑ p-pRb	-	-	-	+

For functional and structural analyses, the scores represent:

- (CTL or equal CTL values);

+ (significantly different *vs*. CTL; one-way ANOVA using Tukey's post-test);

++ (significantly different *vs.* CTL and DEX 0.1; one-way ANOVA using Tukey's post-test);

+++ (significantly different *vs.* CTL, DEX 0.1 and DEX 0.5; one-way ANOVA using Tukey's post-test).

For transductional analysis, the scores represents:

- (CTL or equal CTL values);

+ (at least one component significantly different *vs.* CTL; one-way ANOVA using Tukey's post-test);

++ (all components significantly different *vs.* CTL; one-way ANOVA using Tukey's post-test).

Table 2 Quantification of beta-cell death in control (CTL) and dexamethasone-treated rats (DEX 0.1, DEX 0.5 and DEX 1.0).

	CTL	DEX 0.1	DEX 0.5	DEX 1.0
TUNEL-(+) beta cells (%)	0.19 ± 0.06	0.12 ± 0.05	0.16 ± 0.07	0.15 ± 0.06

Data are means ± S.E.M.

FIGURE LEGENDS

Fig. 1 *Dexamethasone treatment induces decreases in insulin sensitivity and increases in insulin secretion.* Administration of different doses of dexamethasone for five days significantly decreased insulin sensitivity in a dose-dependent fashion **(A)**. Conversely, serum insulin **(B)** and GSIR **(D)** increased significantly in all DEX-treated rats. Blood glucose reached moderate hyperglycemic values only in DEX 1.0 rats **(C)**. Data are means ± S.E.M. [a]significantly different *vs* CTL, [b]significantly different *vs* DEX 0.1, [c] significantly different *vs* DEX 0.5. $n = 6$ for figure A, 10 for figures B and C, and 12 for figure C; $P < 0.05$ for one-way ANOVA using Tukey's post-test.

Fig. 2 *Islet number and beta-cell mass increases in DEX 1.0 rats.* Representative images of pancreas section stained for insulin and hematoxylin **(A)**. Pancreas weight is reduced in DEX 0.1, DEX 0.5 and DEX 1.0 rats **(B)**. DEX 0.5 and 1.0 rats exhibit significant increase in islet number and the relative beta-cell mass **(C, D)**. Absolute beta-cell mass (pancreas weight multiplied by relative beta-cell mass) was significantly altered in insulin-resistant DEX 1.0 rats **(E)**. Data are means ± S.E.M. [a]significantly different *vs* CTL, [b]significantly different *vs* DEX 0.1, [c] significantly different *vs* DEX 0.5. $n = 8$; $P < 0.05$ for one-way ANOVA using Tukey's post-test. Scale bars = 200µm.

Fig. 3 *Insulin-resistant rats exhibit increases in beta-cell size and proliferation.* Representative images of islets stained for PCNA and insulin show increased numbers of positive nuclei for PCNA in beta cells for DEX 0.5 and DEX 1.0 rats **(A)**. The hyperinsulinemic rats (DEX 0.5) showed significant increases in beta-cell size and

proliferation *vs* CTL islets **(B-C)**. DEX 1.0 rats, which present moderate hyperglycemia and high hyperinsulinemia, showed marked increase in beta-cell proliferation and modest increase in beta-cell size **(B-C)**. Data are means ± S.E.M. [a]significantly different *vs* CTL, [b]significantly different *vs* DEX 0.1, [c]significantly different *vs* DEX 0.5. $n \cong 5{,}200$ and 3,500 nuclei of beta cells for beta-cell size and proliferation, respectively; $P < 0.01$ and 0.001 for beta-cell size and proliferation, respectively, for one-way ANOVA using Tukey's post-test. Scale bars = 100 µm.

Fig. 4 *Increases in protein levels of PI3K pathway in islet lysates from DEX 0.5 and DEX 1.0 rats.* DEX 1.0 rats showed significant increases in IRS2, PI3K, phospho-AKT, p70S6K and PDX1 protein content. The protein levels of phospho-AKT and PDX1 were significantly increased in DEX 0.5 rats. DEX 0.1 rats did not exhibit statistically significant differences in protein expression, but tended to increase in IRS2 and phospho-AKT protein levels **(A)**. The figures are representative immunoblots performed at least six times on separate islet extracts. Below the panel, a representative control blot for alpha-tubulin is shown. Data are means ± S.E.M. [a]significantly different *vs* CTL, [b]significantly different *vs* DEX 0.1. $P < 0.05$ for one-way ANOVA using Tukey's post-test. In **(B)** are the representative images of pancreas sections stained for PDX1 and Hematoxylin, showing the wide distribution of PDX1 in pancreatic islets from all experimental groups. Scale bars = 140µm.

Fig. 5 *Increased content of proteins that control the G_1 to S entry phase in islet lysates from DEX 0.5 and DEX 1.0 rats.* DEX 1.0 rats showed significant increases in cyclin D_2, CDK4, phospho-pRb and PCNA and a significant decrease in pRb protein content. Significant increases in protein levels of CDK4 and PCNA, and apparent increases in cyclin D_2 and

phospho-pRb, were noted in DEX 0.5 rats. Protein expression in DEX 0.1 was similar to that of CTL islets.The figures are representative immunoblots performed at least six times on separate islet extracts. Below the panel, a representative control blot for alpha-tubulin is shown. Data are means ± S.E.M. [a]significantly different *vs* CTL, [b]significantly different *vs* DEX 0.1. $P < 0.05$ for one-way ANOVA using Tukey's post-test.

FIGURE 1

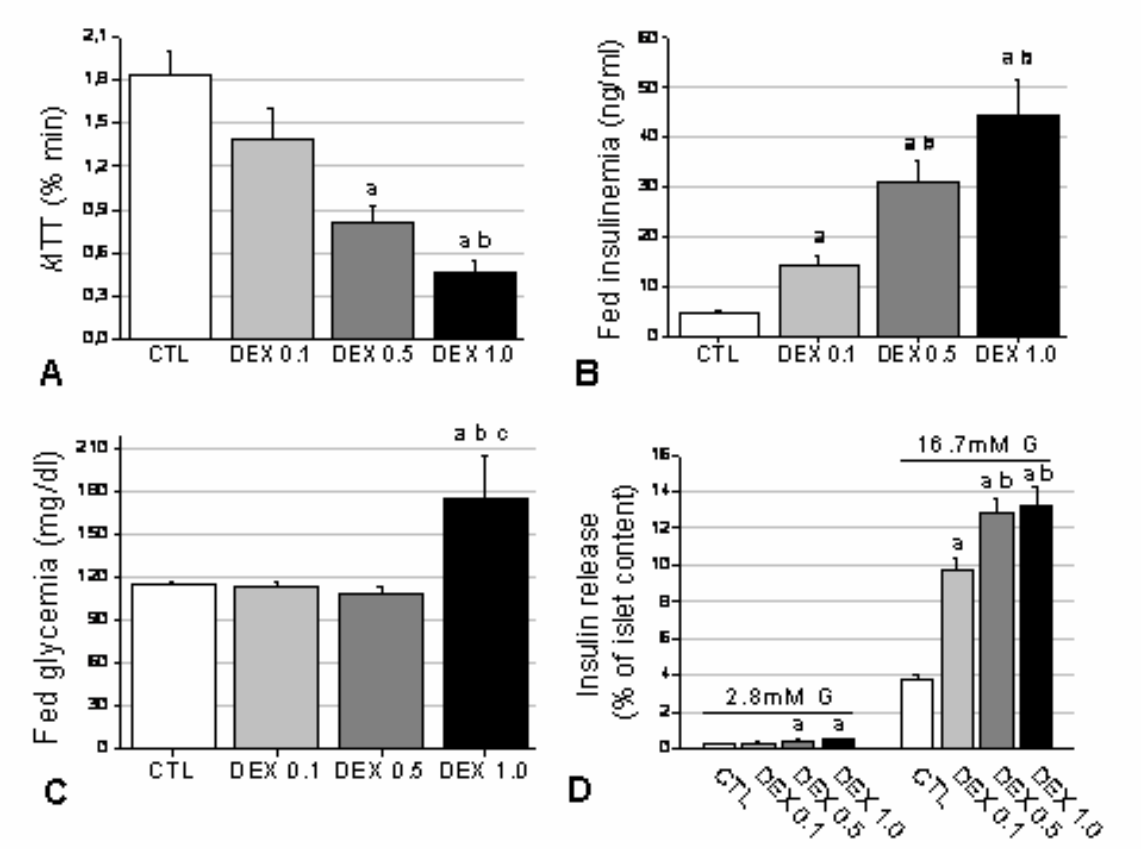

kITT (% min)
2,1
1,8
1,5
1,2
0,9
0,6
0,3
0,0
a
a b
CTL
DEX 0.1
DEX 0.5
DEX 1.0
A
Fed insulinemia (ng/ml)
a
a b
a b
CTL
DEX 0.1
DEX 0.5
DEX 1.0
B
Fed glycemia (mg/dl)
210
180
150
120
90
60
30
0
a b c
CTL
DEX 0.1
DEX 0.5
DEX 1.0
C
Insulin release
(% of islet content)
16
14
12
10
8
6
4
2
0
16.7mM G
a
a b
a b
2.8mM G
a
a
CTL
DEX 0.1
DEX 0.5
DEX 1.0
CTL
DEX 0.1
DEX 0.5
DEX 1.0
D

FIGURE 2

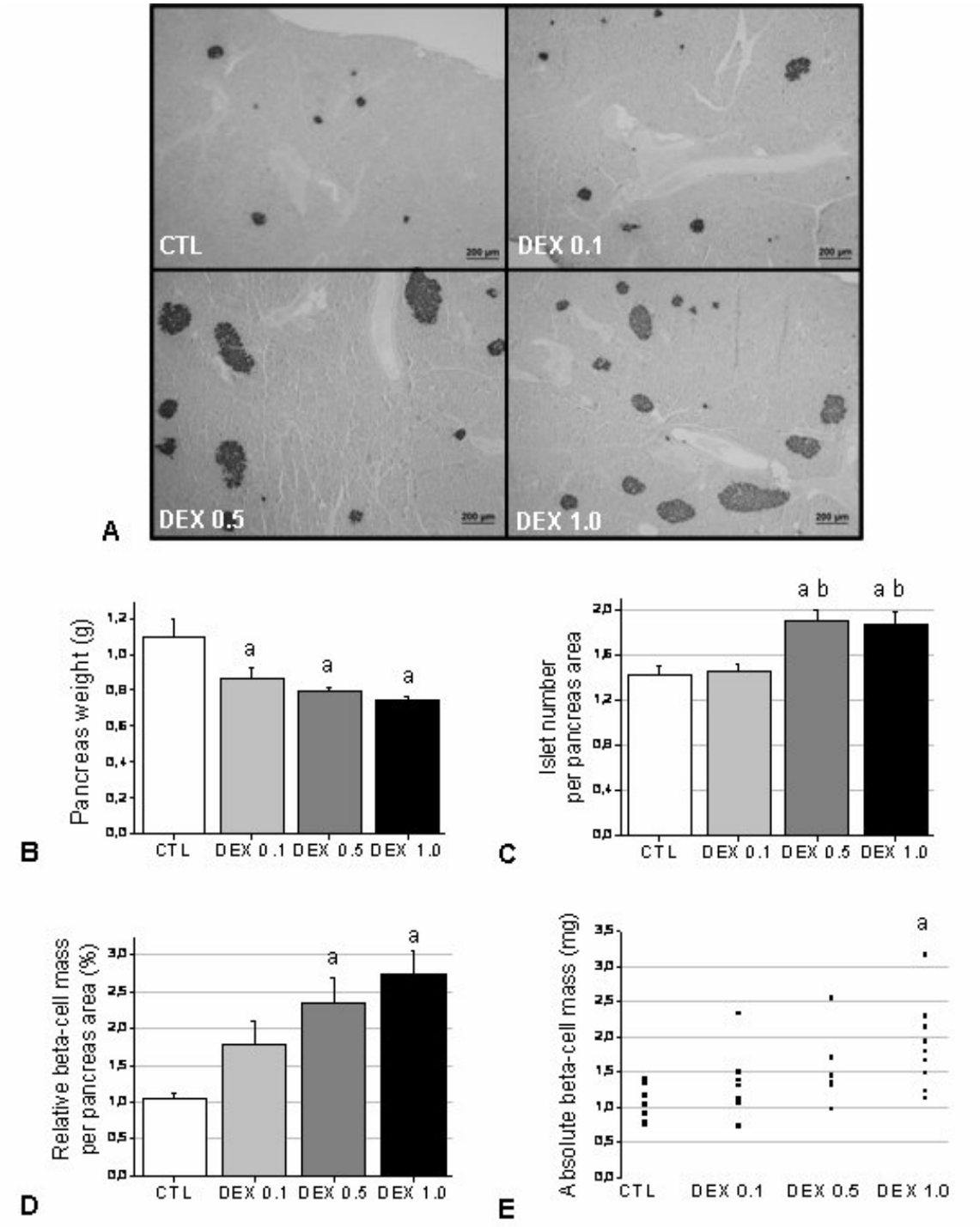

A
CTL
DEX 0.1
DEX 0.5
DEX 1.0
B
Pancreas weight (g)
a
a
a
CTL
DEX 0.1
DEX 0.5
DEX 1.0
C
Islet number per pancreas area
a b
a b
CTL
DEX 0.1
DEX 0.5
DEX 1.0
D
Relative beta-cell mass per pancreas area (%)
a
a
CTL
DEX 0.1
DEX 0.5
DEX 1.0
E
Absolute beta-cell mass (mg)
a
CTL
DEX 0.1
DEX 0.5
DEX 1.0

FIGURE 3

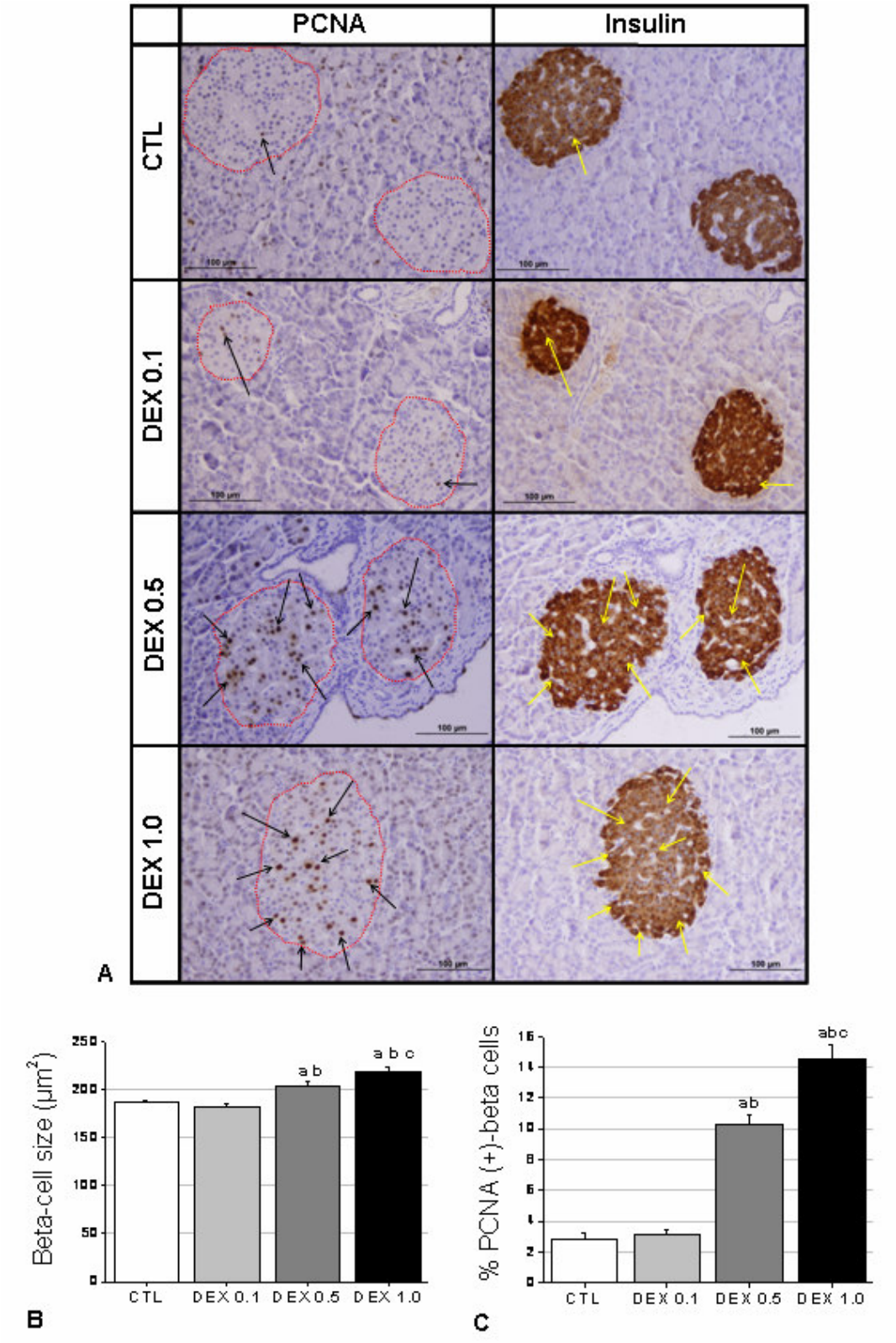
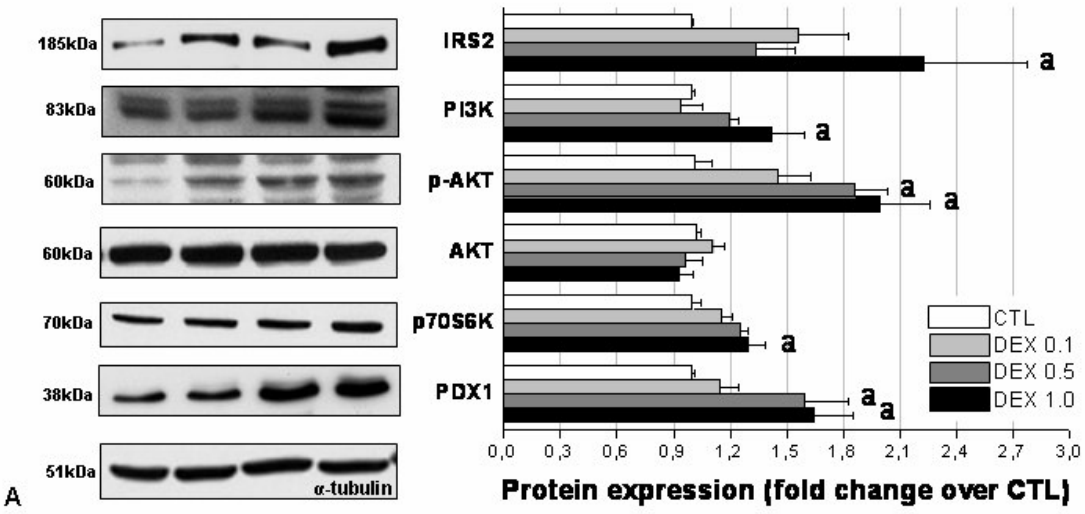

PCNA
Insulin
CTL
DEX 0.1
DEX 0.5
DEX 1.0
A
Beta-cell size (µm²)
a b
a b c
CTL
DEX 0.1
DEX 0.5
DEX 1.0
B
% PCNA (+)-beta cells
ab
abc
CTL
DEX 0.1
DEX 0.5
DEX 1.0
C

FIGURE 4


185kDa
83kDa
60kDa
60kDa
70kDa
38kDa
51kDa
α-tubulin
A
IRS2
PI3K
p-AKT
AKT
p70S6K
PDX1
a
CTL
DEX 0.1
DEX 0.5
DEX 1.0
0,0 0,3 0,6 0,9 1,2 1,5 1,8 2,1 2,4 2,7 3,0
Protein expression (fold change over CTL)

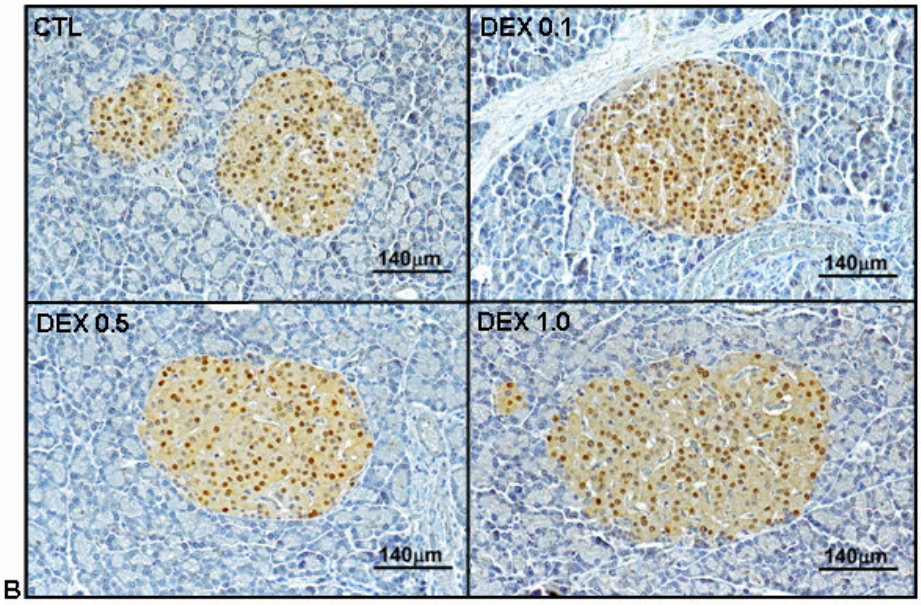
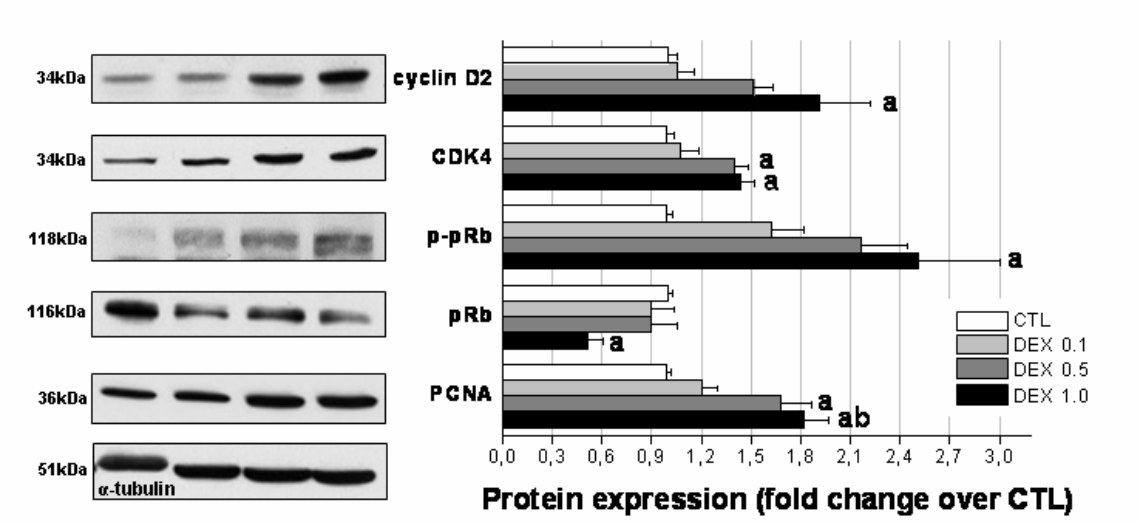

CTL
DEX 0.1
DEX 0.5
DEX 1.0
140μm
B

FIGURE 5


34kDa
34kDa
118kDa
116kDa
36kDa
51kDa
α-tubulin
cyclin D2
CDK4
p-pRb
pRb
PCNA
a
a
a
a
a
ab
CTL
DEX 0.1
DEX 0.5
DEX 1.0
0,0 0,3 0,6 0,9 1,2 1,5 1,8 2,1 2,4 2,7 3,0
Protein expression (fold change over CTL)

Os resultados obtidos nesse trabalho nos levam a concluir que:

- a administração de DEX após 5 dias consecutivos induz RI de maneira dose-dependente, correlacionando-se positivamente com os níveis circulantes de insulina;

- o aumento da secreção de insulina observada *in vivo* em todos os grupos DEX é decorrente do aumento da sensibilidade à glicose pelas ilhotas pancreáticas;

- o aumento dos níveis de insulina sérica observado nos grupos DEX 0.5 e DEX 1.0 pode ter contribuição de mecanismos independentes do metabolismo a julgar pelos experimentos *ex vivo* com Cch e KCl;

- nos animais onde a RI é mais intensa (DEX 0.5 e DEX 1.0) ocorre aumento da proliferação e do tamanho das células β. Estas alterações são maiores nos animais que apresentam marcante hiperinsulinemia juntamente com hiperglicemia moderada (DEX 1.0);

- a alteração do crescimento de células β parece ser dependente da ativação da via IRS-2/Akt/PI3-K/$p70^{S6K}$, bem como da expressão ciclina D_2 e Cdk4 e da fosforilação de pRb;

Portanto, administração de diferentes concentrações de DEX, induz diferentes graus de RI e, por sua vez, diferentes níveis de insulina circulante. Assim, estes modelos tornam-se interessantes para a investigação de mecanismos compensatórios adaptativos que precedem o desenvolvimento do DMT2. Dependendo do grau de RI, pode-se dissociar a influência da hiperglicemia e hiperinsulinemia (DEX 1.0) do da hiperinsulinemia apenas (DEX 0.5 e DEX 0.1) sobre estes parâmetros. Esses dados abrem perspectivas para verificar se as alterações funcionais e estruturais nas ilhotas pancreáticas descritas aqui são decorrentes de efeitos diretos da dexametasona e/ou indiretos, causados pela alteração nos níveis de fatores circulantes resultante da instalação da RI imposta pela administração de DEX.

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ANEXOS

ARTIGO 1

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Dexamethasone-induced insulin resistance is associated with increased connexin 36 mRNA and protein expression in pancreatic rat islets

A. Rafacho, L.P. Roma, S.R. Taboga, A.C. Boschero, and J.R. Bosqueiro

Abstract: Augmented glucose-stimulated insulin secretion (GSIS) is an adaptive mechanism exhibited by pancreatic islets from insulin-resistant animal models. Gap junction proteins have been proposed to contribute to islet function. As such, we investigated the expression of connexin 36 (Cx36), connexin 43 (Cx43), and the glucose transporter Glut2 at mRNA and protein levels in pancreatic islets of dexamethasone (DEX)-induced insulin-resistant rats. Study rats received daily injections of DEX (1 mg/kg body mass, i.p.) for 5 days, whereas control rats (CTL) received saline solution. DEX rats exhibited peripheral insulin resistance, as indicated by the significant postabsorptive insulin levels and by the constant rate for glucose disappearance (K_{ITT}). GSIS was significantly higher in DEX islets (1.8-fold in 16.7 mmol/L glucose vs. CTL, $p < 0.05$). A significant increase of 2.25-fold in islet area was observed in DEX vs. CTL islets ($p < 0.05$). Cx36 mRNA expression was significantly augmented, Cx43 diminished, and Glut2 mRNA was unaltered in islets of DEX vs. CTL ($p < 0.05$). Cx36 protein expression was 1.6-fold higher than that of CTL islets ($p < 0.05$). Glut2 protein expression was unaltered and Cx43 was not detected at the protein level. We conclude that DEX-induced insulin resistance is accompanied by increased GSIS and this may be associated with increase of Cx36 protein expression.

Key words: connexins, glucocorticoids, insulin resistance, glucose-stimulated insulin secretion, pancreatic islets.

Résumé : L'augmentation de la sécrétion d'insuline stimulée par le glucose (SISG) est un mécanisme adaptatif montré par les îlots pancréatiques de modèles animaux insulinorésistants. On croit que les protéines des jonctions lacunaires participent à la fonction des îlots. Pour vérifier cette hypothèse, nous avons examiné l'expression de la connexine 36 (Cx36), de la connexine 43 (Cx43) et de Glut2 au niveau de l'ARNm et au niveau protéique dans les îlots pancréatiques de rats rendus insulinorésistants par dexaméthasone. Nous avons soumis des rats (DEX) à des injections quotidiennes de dexaméthasone (1 mg/kg, p.c., i.p.) pendant 5 jours, alors que les rats témoins (CTL) ont reçu une solution saline. Les rats DEX ont montré une insulinorésistance périphérique, comme indiqué par les taux élevés d'insuline en phase postabsorptive et par le test de tolérance à l'insuline (valeur K_{ITI}). La SISG a été beaucoup plus élevée dans les îlots DEX (facteur 1,8 dans 16,7 mmol/L de glucose vs CTL; $p < 0,05$). Une augmentation significative d'un facteur 2,25 de la surface des îlots a été observée dans les îlots DEX vs CTL ($p < 0,05$). L'expression de l'ARNm Cx36 a augmenté de manière significative, Cx43 a diminué et l'ARNm Glut2 est demeuré stable dans les îlots DEX vs CTL ($p < 0,05$). L'expression de la protéine Cx36 a été d'un facteur 1,6 plus élevée que dans les îlots CTL ($p < 0,05$). L'expression protéique de Glut2 n'a pas été modifiée, et Cx43 n'a pas été détectée à ce niveau. Nous concluons que l'insulinorésistance induite par le dexaméthasone est accompagnée d'une augmentation de la SISG et que cette association pourrait être liée à une augmentation de l'expression protéique de Cx36.

Mots-clés : connexines, glucocorticoïdes, insulinorésistance, sécrétion d'insuline stimulée par le glucose, îlots pancréatiques.

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Introduction

Glucocorticoids are widely used in clinical practice. These hormones can induce insulin resistance and, depending on the dose and time of treatment, can cause type 2 diabetes (Beard et al. 1984; Hoogwerf and Danese 1999; Pagano et al. 1983; Perley and Kipnis 1966). Several studies have demonstrated the capacity of dexamethasone (DEX) to induce peripheral insulin resistance either in vivo (Barbera et al. 2001; Nicod et al. 2003; Saad et al. 1993; Severino et al. 2002) or in vitro (Burén et al. 2002; Ruzzin et al. 2005). Decreased glucose uptake by the peripheral tissues and increased hepatic glucose output are the basis of insulin

resistance. The major aspects that have been associated with this (patho)physiology are normoglycaemia or moderate hyperglycaemia, together with hyperinsulinaemia (Barbera et al. 2001; Nicod et al. 2003; Severino et al. 2002), a compensatory increase in β-cell mass (Ogawa et al. 1992), an increase in total pancreas insulin content (Bonner-Weir et al. 1981), and augmented glucose-stimulated insulin secretion (GSIS) (Holness et al. 2005; Novelli et al. 1999).

The latter function has been extensively investigated in pancreatic islets. Insulin secretion is a multicellular process controlled by several factors and is crucial for the control of blood nutrient concentrations, especially glucose. A tightly regulated interaction between the pancreatic islet cells is necessary to coordinate the proper insulin secretion from hundreds of islets (Porksen et al. 2002). In pancreatic islets, the communication between β cells and non-β cells is of fundamental importance for normal islet function (Bosco and Meda 1991), since it has been demonstrated that dissociated islet cells and single purified β cells exhibit decreased secretory activity compared with intact islets. This phenomenon occurs mainly as a result of diminished paracrine effects among islet cells (Pipeleers 1984) together with impairment of electrical activity (e.g., synchronization of Ca^{2+} oscillations among β cells) (Kanno et al. 2002).

One of the most studied intercellular junctions is the gap junction. These junctions are intercellular channels that directly connect the cytoplasm of neighboring cells, thus allowing the exchange of nucleotides, inorganic ions, and small molecules <1.2 kDa. Gap junction intercellular communications (GJICs) are formed by the apposition of 2 hemichannels, termed connexons, composed of 6 protein subunits known as connexins, forming a central hydrophilic pore (Saez et al. 2003). The presence of gap junctions between β cells is required for proper control of insulin secretion. Thus, single β cells, which are not coupled by intercellular channels, show poor insulin gene expression, as indicated by the reduction observed in total RNA (Philippe et al. 1992), and release small amounts of the hormone under normal and stimulation conditions (Bosco and Meda 1991). However, insulin biosynthesis and release are rapidly improved after reaggregation of β cells and its contacts (Meda et al. 1990; Meda 1996).

It has been reported that the Cx36 protein is the sole connexin isoform observed in pancreatic β cells (Serre-Beinier et al. 2000; Theis et al. 2004). Increased or decreased Cx36 content in insulin-producing cells, generated by cell transfection techniques, affect the ability of these cells to respond to glucose (Calabrese et al. 2003; Calabrese et al. 2004; Le Gurun et al. 2003). In addition, pharmacological agents can acutely or chronically affect GJICs (Dhein et al. 2002). These observations demonstrate that gap junction proteins may somehow act on pancreatic β-cell function and are modulated under certain pharmacological conditions. Studies evaluating the contribution of Cx36 to the in vivo function of the endocrine pancreas in pathological experimental animal models are scarce. Thus, the current study may be useful since, to our knowledge, there is no available study to demonstrate the expression of Cx36 and Cx43 in a rat model of insulin resistance that features augmented glucose-stimulated insulin secretion.

Thus, we demonstrate that rats induced to insulin resistance after 5 days of DEX administration (daily injection of 1 mg/kg body mass) exhibit increased glucose-stimulated insulin secretion and this is associated with increased gap junction protein Cx36 mRNA and protein expression.

Materials and methods

Materials

Dexamethasone phosphate (Decadron®) was from Aché (Campinas, SP, Brazil). Sodium thiopental (Thiopentax®) was from Cristália (Itapira, SP, Brazil). Human recombinant insulin (Biohulin® N) was from Biobrás (Montes Claros, MG, Brazil). Dextrose, NaCl, KCl, $CaCl_2$, $MgCl_2$, $NaHCO_3$, KOH, and Na_2SO_4 were from Mallinckrodt Baker, Inc. (Paris, France). Collagenase, HEPES, albumin, activated charcoal, and dextran were from Sigma (St. Louis, Mo.). Ethanol, methanol, chloroform, and phenol were from Synth (Diadema, SP, Brazil). SDS-PAGE and immunoblotting were performed using Bio-Rad systems (Hercules, Calif.). All chemicals used for immunoblotting were from Sigma (St. Louis, Mo.). All reagents used in the experiments for RT-PCR were from Invitrogen (Carlsbad, Calif.). Anti-Cx36 (goat polyclonal), anti-Cx43 (rabbit polyclonal), and anti-Glut2 (rabbit polyclonal) antibodies were from Santa Cruz Biotechnology, Calif.

Animals and DEX treatment

Experiments with animals were approved by the institutional (UNESP) Committee for Ethics in Animal Experimentation and conform to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH publication No. 85-23 revised 1996).

Groups of 5 male Wistar rats (approx. 90 days old) from the breeding colony at UNESP were kept at 24 °C on a 12 h light : 12 h dark cycle. The rats had free access to food and water. Dexamethasone-treated (DEX) rats received a daily injection (1 mg/kg body mass, i.p.) for 5 days, whereas control (CTL) rats received saline solution (NaCl 0.9%, 1 mL/kg body mass, i.p.) for 5 days.

Metabolic, hormonal, and biochemical measurements

Rats were weighed before the onset of treatment and on the day following the last DEX administration. On the day following the last DEX administration, fasted (12–14 h) rats were sacrificed by exposure to CO_2, which was followed by decapitation. Blood glucose concentration was measured with a glucometer (OneTouch, Johnson & Johnson, Milpitas, Calif.). Serum was obtained by 1 h incubation at 37 °C and centrifugation at 430*g*, and insulin levels were detected by radioimmunoassay (RIA) utilizing a rabbit anti-rat insulin antibody and rat insulin as standard (Scott et al. 1981).

Glycogen and fat measurements in liver

Determination of hepatic glycogen was performed according to Lo et al. (1970) with some modifications. Briefly, the liver samples (300 to 500 mg) were transferred to test tubes containing 30% KOH (*w*/*v*) and boiled for 1 h until complete homogenization. Na_2SO_4 was then added and the glycogen was precipitated with ethanol. The samples were centrifuged at 800*g* for 10 min, the supernatants discarded, and the glycogen dissolved in hot distilled water. Ethanol

was added and the pellets, obtained after a second centrifugation, were dissolved in distilled water in a final volume of 20 mL. Glycogen content was measured by treating a fixed volume of the samples with phenol reagent and H_2SO_4, and the absorbance was then read at 490 nm with a spectrophotometer.

Intraperitoneal glucose tolerance test (ipGTT)

On the day following the last DEX-administration, each group of fasted (12–14 h) rats was anaesthetized with sodium thiopental (60 mg/kg body mass, i.p.). After checking for the absence of corneal and pedal reflexes, an unchallenged sample (time 0) was obtained from the tail. Immediately thereafter, 50% glucose (2 g/kg body mass, i.p.) was administered. Blood samples were collected at 30, 60, 90, and 120 min from the tail tip for determination of glucose concentrations.

Intraperitoneal insulin tolerance test (ipITT)

Fed rats were anaesthetized as described above. Human recombinant insulin (equivalent to 2 U/kg body mass, i.p.) was injected as soon as the glucose concentration from the tail tip was determined at time 0. Further samples were collected at 5, 10, 20, and 30 min for measurement of glycaemia. The constant rate for glucose disappearance (K_{ITT}) was calculated from the slope of the regression line obtained with log-transformed glucose values between 0 and 30 min after insulin administration.

Isolation of islet, insulin content, and static secretion protocols

Islets were isolated by collagenase digestion of the pancreas as described (Lacy and Kostianovsky 1967) with modifications (Boschero et al. 1980). Insulin contents of islets were determined after extraction in acid-ethanol solution. For static incubation, groups of 5 islets were first incubated for 1 h at 37 °C in a Krebs bicarbonate buffer solution of the following composition (in mmol/L): 115 NaCl, 5 KCl, 2.56 $CaCl_2$, 1 $MgCl_2$, 24 $NaHCO_3$, 15 HEPES, and 5.6 glucose, supplemented with 0.5% of bovine serum albumin and equilibrated with a mixture of 95% O_2 : 5% CO_2, pH 7.4. The medium was then replaced with fresh buffer containing the following solutions of glucose (in mmol/L): 2.8, 5.6, 8.3, 11.1, 16.7, and 22 and incubated for another 1 h. At the end of the incubation, the test plates were left in a refrigerator drawer for 15 min before the supernatant was collected and appropriately stored at –20 °C for subsequent measurement of insulin content by RIA as described earlier.

Histomorphologic and histomorphometric analysis

Histologic sections stained by Gömori's trichrome were employed to examine the morphological aspects of the islets (Bancroft and Stevens 1990). To determine the area, pools of 500 islets of each group were submitted to Feulgen's DNA method en bloc (Mello 1997). The islet images were then registered by a CCD camera and area (μm^2) values were automatically obtained by the Image-Pro-Plus® Media, Cybernetics program, coupled to a BX-60 Olympus photomicroscope.

Tissue extraction and immunoblotting

Pools of at least 1000 islets were homogenized in 150 µL of ice-cold buffer containing 10 mmol/L imidazole, 4 mmol/L EDTA, 1 mmol/L EGTA, 200 µg/mL pepstain, 200 kIU/mL aprotinin, 200 µmol/L PMSF, 2.5 µg/mL leupeptin, 30 µg/mL trypsin-inhibitor of protease inhibitors, pH 7.4, and 1% SDS and 1% Triton X-100. Homogenates were rotated for 20 s at 4 °C by using a Polytron PT 1200C homogenizer (Brinkmann Instruments, N.Y.). After centrifugation for 20 min at 12 000*g* and 4 °C, the protein concentration in supernatants was determined by the Bradford method. For electrophoresis, aliquots of 50 µg of protein were incubated for 60 min at 37 °C with 25% of the total volume with 5× concentrated Laemmli sample buffer (1 mol/L sodium phosphate pH 7.0, 10% SDS, 10% β-mercaptoethanol, 50% glycerol, and 0.1% bromophenol blue). The samples were fractioned by electrophoresis in 10% SDS–PAGE. Transfer of proteins from the gel onto nitrocellulose membranes was performed for 1 h at 120 V in the presence of 20% methanol. After checking for transfer efficiency by Ponceau S staining, the membranes were saturated with 5% dried skimmed milk in TBST (10 mmol/L Trizma base, 150 mmol/L NaCl, 0.05% Tween 20) overnight at 4 °C. After blocking, membranes were washed in TBST (3 × 7 min) and incubated for 2 h with the appropriate primary antibody at room temperature (RT) in TBST plus 3% dry skimmed milk. After washing in TBST (3 × 10 min), membranes were incubated with the appropriate secondary antibody conjugated with horseradish peroxidase for 90 min in TBST plus 1% dried skimmed milk at RT, and washed again. Antibody binding was detected by enhanced SuperSignal® West Pico Chemiluminescent Substrate (Pierce, Rockford, Ill.), as described by the manufacturer. Blots were scanned (Epson expression 1600) and signals quantified by densitometry with Scion Image software (Scion Corporation, Frederick, Md.).

RNA isolation and semiquantitative RT-PCR analysis

Total RNA from islets of 10–12 rats of each group was extracted using Trizol reagent as described by the manufacturer. For the polymerase chain reaction (PCR), RNA was reverse-transcribed using random primers. The resulting cDNA was amplified by RT-PCR using oligonucleotides complementary to the sequences of the Cx36 gene (GenBank acc. No. AJ296282); sense 5′-AGTGGTGGGAGCAAGCGAGAAG-3′ and antisense 5′-GGCTTCAGTGTCCCAGGGTTGT-3′ primers amplified a 281 bp cDNA fragment. For the Cx43 gene (GenBank acc. No. NM012567); sense 5′-CCGACGACAACCAGAATGCC-3′ and antisense 5′-CTTGGGATAGCTGGGCGGAAC-3′ primers amplified a 321 bp cDNA fragment. For the Glut2 gene (GenBank acc. No. L28126); sense 5′-CACATAGGCTCTCTGCATTG-3′ and antisense 5′-GGAATCAGCCAAAAAGAAGA-3′ primers amplified a 378 bp cDNA fragment. For the RPS29 (ribosomal protein S29) gene (GenBank acc. No. NM012876), sense 5′-AGGCAAGATGGGTCACCAGC-3′ and antisense 5′-AGTCGAATCATCCATTCAGGTCG-3′ primers amplified a 201 bp cDNA fragment used as an internal control. The PCR was performed in a 25 µL reaction volume containing 1 µL of cDNA equivalent to 3 µg of RNA, 10 mmol/L cold dNTPs/L (dATP, dCTP, dGTP, dTTP), 50 mmol/L $MgCl_2$, 10× PCR buffer, 10 µmol/L of appropriate oligonucleotides primers, and 2 U of *Taq* polymerase. The number of cycles was se-

Table 1. Body mass, serum glucose and insulin levels, and liver glycogen and fat content in control and dexamethasone-treated rats, $n = 10$.

Parameters	CTL	DEX	Rate DEX/CTL
Body mass (g)	411.2±13.3	357.8±8.9	0.87
Glucose (mg/dL)	95±2.4	151±19.*	1.58
Insulin (ng/mL)	0.82±0.14	5.15±0.3*	6.28
Liver glycogen (g/100 g)	1.9±0.3	5.2±0.1*	2.73
Liver fat (g/100 g)	5.4±0.2	10.7±2.2*	1.98

Note: CTL, control; DEX, dexamethasone. Values are means ± SE. *$p < 0.05$ vs. control.

Fig. 1. Insulin intolerance occurs after dexamethasone (DEX) treatment in vivo. DEX rats demonstrated marked reduction in the constant rate for glucose disappearance (K_{ITT}) during the intraperitoneal insulin tolerance test (ipITT). Data are means ± SE. *$p < 0.05$ vs. control (CTL), $n = 6$.

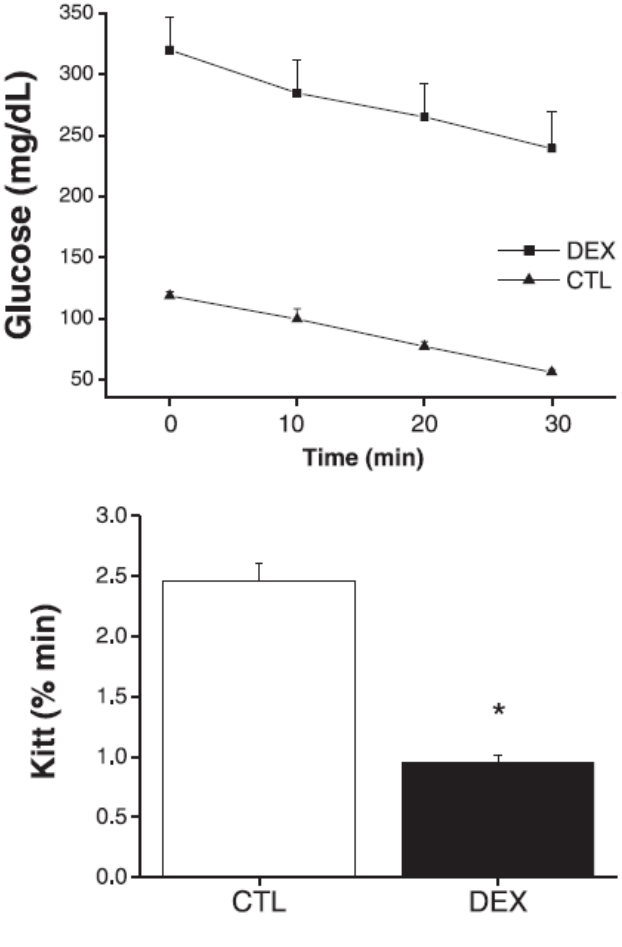
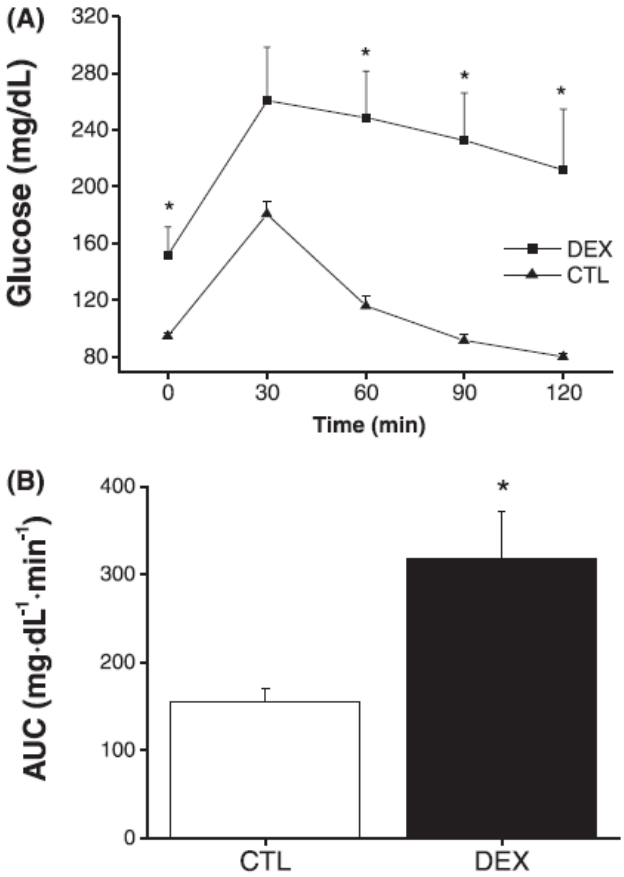
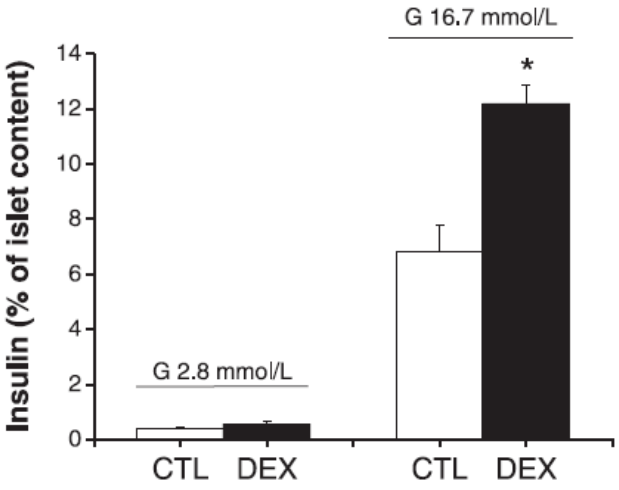


Fig. 2. Dexamethasone (DEX) rats exhibited glucose intolerance after a glucose challenge. Rats were treated with intraperitoneal injections of DEX (1 mg/kg) or saline (CTL) for 5 days. (A) Rats received glucose (2 g/kg, i.p.), and blood samples were obtained from the cut tip of the tail at 0, 30, 60, 90, and 120 min for glucose measurements. (B) Area-under-the-curve (AUC) data illustrated increment in DEX 1.0. Data are means ± SE. *$p < 0.05$ vs. control, $n = 6$.



lected to allow linear amplification of the cDNA under study. The PCR conditions for amplification of Cx36, Cx43, Glut2, and RPS29 genes were: 2 min at 94 °C followed by 29 cycles at 94 °C (30 s), 60 °C at 30 s, and 72 °C at 45 s; 2 min at 94 °C followed by 29 cycles at 94 °C (30 s), 57 °C at 30 s, and 72 °C at 45 s; 2 min at 94 °C followed by 23 cycles at 94 °C (30 s), 55 °C at 30 s, and 72 °C at 45 s; and 2 min at 94 °C followed by 27 cycles at 94 °C (30 s), 57 °C at 30 s, and 72 °C at 45 s, respectively. The PCR products were separated on 1.5% agarose gel in 1× TBE buffer and stained with ethidium bromide. All PCR reactions included a negative control. The absence of genomic contamination in the RNA samples was confirmed by the RT-negative RNA samples. Subsequent digitalization and measurement of relative band intensities were performed employing a Gel Doc documentation system (BioRad Laboratory, Hercules, Calif.). The results were expressed as a ratio be-

Fig. 3. Glucocorticoid treatment in vivo increases glucose-induced insulin release. At a glucose concentration of 2.8 mmol/L, insulin secretion was not different between dexamethasone (DEX) and control (CTL) islets. At a stimulatory glucose concentration, the insulin secretion was enhanced in DEX islets (1.8-fold higher vs. CTL islets). Data are means ± SE. *$p < 0.05$ vs. CTL, $n = 12$.



tween the target gene amplification and internal control product RPS-29.

Statistical analysis

Results are presented as means ± SE. Statistical comparisons between data from DEX and CTL groups were performed using the unpaired Student's t test. The level of significance was set at $p < 0.05$.

Results

Metabolic status, ipITT, and ipGTT

Table 1 shows the mean values of mass, glycaemia, insulinaemia, and hepatic glycogen and hepatic fat content of fasted rats. DEX rats demonstrated a significant reduction in body mass and a significant postabsorptive increase in blood glucose and insulin levels and hepatic glycogen and hepatic fat content compared with CTL rats ($n = 10$, $p < 0.05$). An ipITT was performed to detect possible perturbations in peripheral insulin sensitivity. DEX rats exhibited insulin intolerance, expressed by the reduction in the constant rate for glucose disappearance (K_{ITT}) as indicated in Fig. 1 ($n = 6$, $p < 0.05$). Glucose disappearance was 2.6-fold slower in DEX than in CTL rats. Figure 2 shows the impairment of peripheral glucose tolerance in DEX rats during the ipGTT experiment. Plasma glucose of DEX rats remained elevated at more than 200 mg/dL at least 2 h after glucose administration. Blood glucose level was statistically different from that of the CTL before and 60, 90, and 120 min after glucose loading ($n = 6$, $p < 0.05$). Integrated area-under-the-curve (AUC) data depicted an increase in the DEX group compared with CTL ($p < 0.05$) (Fig. 2).

DEX treatment in vivo increases GSIS ex vivo

After normalization by the total islet insulin content, insulin secretion in DEX islets was not different from that observed in CTL islets at a concentration of 2.8 mmol/L glucose. At 16.7 mmol/L glucose, the insulin secretion in DEX islets was 1.8-fold higher than that in CTL islets (12.2% ± 0.7% and 6.8% ± 0.9% of total insulin content for DEX and CTL islets, respectively, $n = 12$, $p < 0.05$; Fig. 3). The dose–response curve to glucose (2.8–22 mmol/L) was significantly shifted to the left in DEX rat islets compared with CTL islets (not shown) with an EC_{50} of 7.3 and 9.3 mmol/L glucose, respectively, ($p < 0.05$), indicating an islet sensitivity to glucose.

Five days of DEX treatment induces hypertrophy of pancreatic islets

After treatment, DEX islets showed a marked hypertrophy compared with CTL islets (Fig. 4). The area values were significantly higher in islets from DEX compared with those of CTL rats (11 114 ± 290 and 25 048 ± 894 μm^2 for CTL and DEX, respectively, $n = 500$ islets, $p < 0.05$).

Increase in Cx36 mRNA and protein expression

A significant increase in Cx36 mRNA of 1.5-fold was observed ($n = 12$, $p < 0.05$; Fig. 5), concomitant with a 1.6-fold increased protein expression ($n = 4$, $p < 0.05$; Fig. 6) in pancreatic islets from DEX rats compared with CTL islets. The increased values of protein Cx36 expression ranged from 20%–80%. A diminished expression of 31.5% ± 6.5% in Cx43 mRNA was observed ($n = 12$, $p < 0.05$; Fig. 5); however, no alteration at the mRNA level was detected for Glut2 compared with CTL islets ($n = 12$, $p < 0.05$; Fig. 5). In addition, at the protein level, Glut2 from DEX islets exhibited no alteration compared with CTL levels ($n = 4$; $p < 0.05$) and Cx43 protein was not expressed in either group of islets ($n = 4$, $p < 0.05$; Fig. 6).

Discussion

We have assessed, for the first time, the expression of Cx36 and Cx43 at the mRNA and protein levels in an experimental model of insulin resistance, induced by the glucocorticoid dexamethasone. One of the main results of the current study is the observation that, in this (patho) physiological condition, which features enhanced GSIS (Weir et al. 2001), there is an increase in Cx36 mRNA and protein expression; Cx36 is the exclusive β-cell gap junction protein in pancreatic islets.

It is well known that glucocorticoids induce peripheral insulin resistance both in vivo and in vitro (Barbera et al. 2001; Burén et al. 2002; Nicod et al. 2003; Ruzzin et al. 2005; Saad et al. 1993; Severino et al. 2002), by increasing hepatic glucose output and decreasing the peripheral glucose uptake. The body mass reduction and the increase in hepatic glycogen production in DEX rats in the present study are in agreement with the literature with regard to glucocorticoid-induced insulin resistance (Caldefie-Chezet et al. 2001; Mokuda et al.1991). The islet hypertrophy and increase in total islet insulin content in DEX rats also corroborate other studies (Bonner-Weir et al. 1981; Ogawa et al. 1992). These compensatory responses, in addition to the increase in post-absorptive circulating insulin levels, are consequences of the peripheral insulin resistance imposed by DEX treatment. Normally, in healthy human subjects, changes in insulin sen-

Fig. 4. Islet hypertrophy occurs in compensation to insulin resistance. Dexamethasone (DEX) treatment causes islet hypertrophy when control (CTL) islets (A and A′) are compared with DEX islets (B and B′). *exocrine tissue; i, islet of Langerhans; v, blood vessel. (A) and (B) are light microscopic views of pancreatic islets submitted to Gömori's trichrome method. Scale bar represents 100 μm. (A′) and (B′) are isolated islets stained by Feulgen's reaction en bloc. Scale bar represents 50 μm.

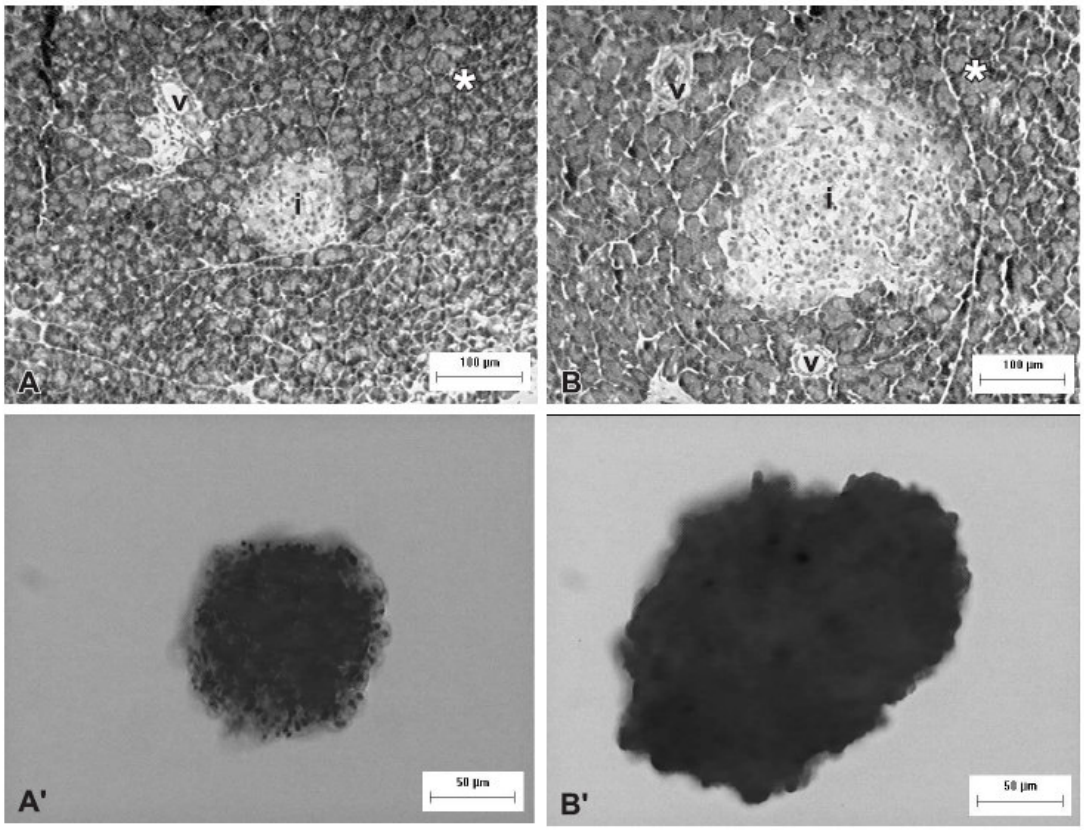
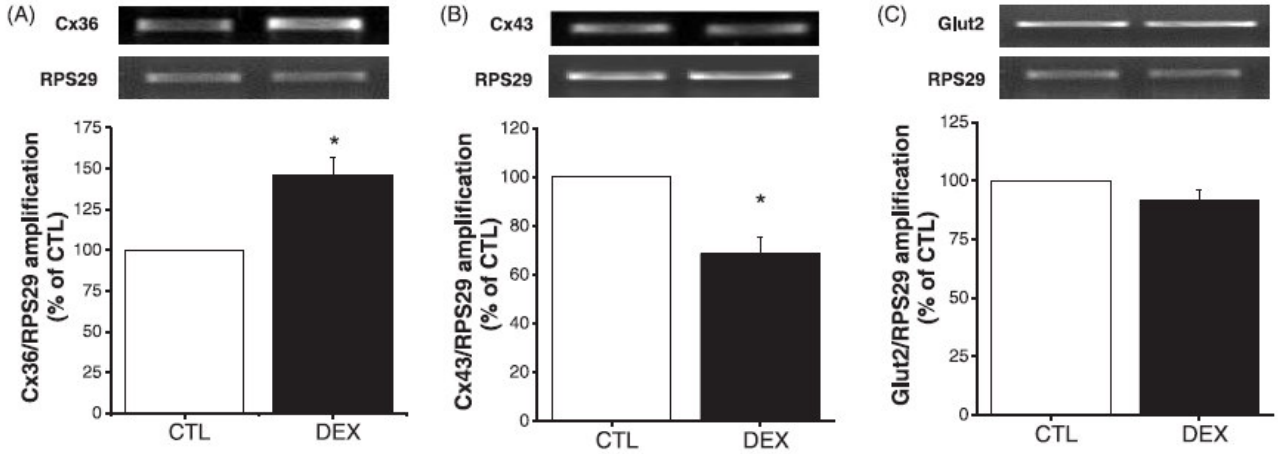
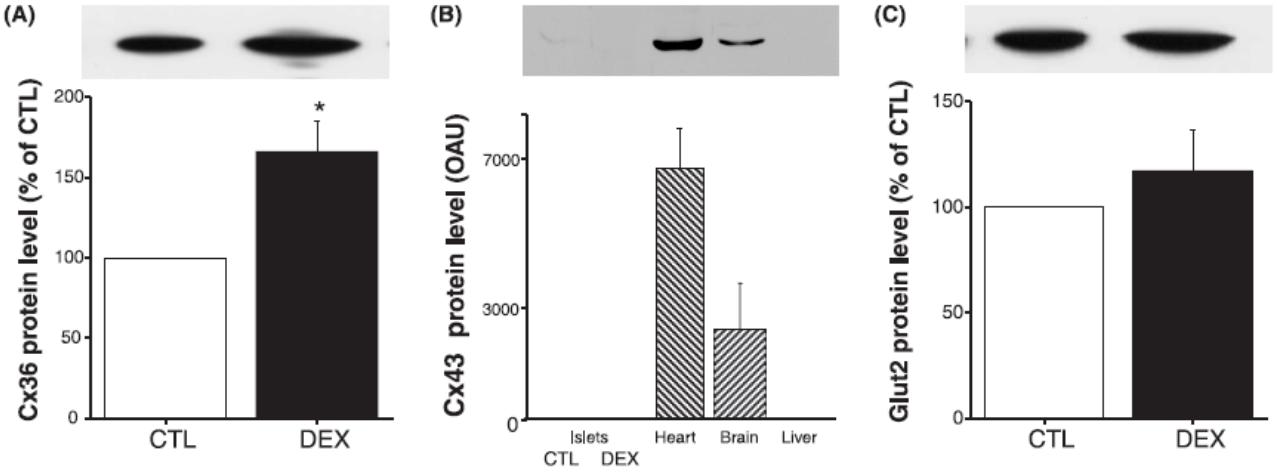


Fig. 5. Increase in Cx36 mRNA expression in dexamethasone (DEX) islets. (A) Cx36 gene fragment of 281 bp, (B) Cx43 gene fragment of 321 bp, and (C) Glut2 gene fragment of 378 bp after running in 1.5% agarose gel. Data are means ± SE of quadruplicates of 3 separated cDNAs for each group. *$p < 0.05$ vs. control (CTL).



sitivity are compensated by reciprocal changes in glucose-stimulated insulin secretion in vivo, such that the product of insulin sensitivity and insulin secretion remains unchanged (Bergman et al. 2002). In our DEX-induced insulin-resistant rats, this observation is consistent, since these rats exhibit significant postabsorptive insulin levels, but not enough to maintain normoglycaemia. Furthermore, the insulin secretion response after glucose loading during the glucose tolerance test was unable to counteract the elevation of glucose concentration. Thus, these rats develop peripheral glucose intol-

Fig. 6. Increase in Cx36 protein expression in dexamethasone (DEX) islets. Extracts applied to 10% polyacrylamide gel and blotted with antibodies: (A) islets blotted with anti-Cx36, (B) islets, heart, brain, and liver blotted with anti-Cx43, and (C) islets blotted with anti-Glut2. Note the absence of Cx43 expression in islets in Fig. 6B. OAU, optical arbitrary units. Data are means ± SE of 4 separate experiments. *$p <$ 0.05 vs. control (CTL).



erance. Previous studies using animal models of insulin resistance such as 18- and 26-month-old rats treated with DEX (Novelli et al. 1999), transgenic mice with increased glucocorticoid sensitivity (Davani et al. 2004), and rats fed on a high-saturated-fat diet together with DEX administration (Holness et al. 2005) fully confirm this inability to prevent glucose intolerance.

We have also demonstrated alterations in GSIS with isolated islets after static incubation. Several authors have studied the insulin release measurement in vitro after glucocorticoid treatment in experimental animals; however, results are controversial. Enhanced (Kawai and Kuzuya 1977; Malaisse et al. 1967; Novelli et al. 1999; Wang et al. 1994), unaltered (Chuthaputti and Fletcher 1987; O'Brien et al. 1991), or reduced (Delaunay et al. 1997; Gremlich et al. 1997; Jeong et al. 2001; Khan et al. 1992; Lambillotte et al. 1997) insulin secretion has been described in either perfused pancreas or isolated islets. It is reasonable to observe that countless parameters may be behind these different results, including the dose and time of glucocorticoid treatment, the strain and model of the animals, and the stimulus applied in vitro (e.g., the choice of glucose concentrations), thus making an interpretation difficult. We showed, after 1 h incubation at 16.7 mmol/L glucose, an enhanced insulin secretion by DEX islets. It has been suggested that, in experimental animal models in which the insulin resistance is present and the islet function is preserved (Novelli et al. 1999; Ogawa et al. 1992), the islets are still able to respond to glucose ex vivo, which is not observed in experimental models of diabetes characterized by diminished postabsorptive insulin levels, marked hyperglycaemia, and decreased GSIS (Ogawa et al. 1992; Toriumi and Imai 2003). In addition, the increase in insulin secretion observed in DEX-treated rat islets following glucose stimulation would imply a degree of islet adaptation (i.e., the sensitization to glucose observed by EC_{50} values) for the impairment of insulin action caused by DEX treatment. Thus, the adaptive compensatory requirements that occur in vivo might exert effects that prevail on the ex vivo stimulus.

Another interesting aspect of our results is the increase in Cx36 mRNA and protein expression in pancreatic islets under this DEX-induced insulin resistance model. It has been proposed that connexins may contribute to the control of several functions of insulin-producing cells and pancreatic islets. An association is observed between the increased expression of Cx43 protein and an increase in insulin release in prolactin-cultured neonatal islets (Collares-Buzato et al. 2001). An association has also been observed between increased mRNA and protein expression of Cx36 and Cx43 and increased insulin secretion following 7 days of islet culturing (Leite et al. 2005). Recent studies, however, have demonstrated reduced or unaltered total insulin content and GSIS in insulin-producing cells, pseudo-islets, and pancreatic islets after induction of Cx36 protein overexpression (Caton et al. 2003; Le Gurun et al. 2003). The same functional responses such as reduced total insulin content and reduction of GSIS were observed when INS-1E cells were transfected with antisense for Cx36, whereas a reduction in total insulin content and unaltered insulin secretion with 16.7 mmol/L glucose was observed in native pancreatic islets transfected with the same antisense (Le Gurun et al. 2003). Loss of Cx36 with stable transfection of an antisense Cx36 cDNA decreased functional gap junctional conductance in MIN6 cell clones. This alteration impaired the synchronization of glucose-induced $[Ca^{2+}]_I$ oscillations and insulin release in response to glucose, to substances that increase $[cAMP]_I$, and to depolarizing conditions (Calabrese et al. 2003; Calabrese et al. 2004). It has been demonstrated that gap junctions are not required for intercellular Ca^{2+} wave oscillation and insulin secretion in response to glucose in whole islets or clusters of islets after treatment with gap junction uncouplers (Bertuzzi et al. 1999; Squires et al. 2000). This finding indicates the existence of a different control of Ca^{2+} dissemination throughout the islets, probably located in the extracellular compartment. Recent data with mice homozygous for the Cx36 deletion ($Cx36^{-/-}$), however, demonstrated that communication via Cx36 channels is a central event in the synchronization of Ca^{2+} oscillations

among β cells within individual pancreatic islets and that disruption of this connexin-dependent synchronization results in the loss of insulin pulses normally elicited by glucose and in increased basal release of the hormone (Ravier et al. 2005).

We have also investigated the expression of Cx43 and Glut2 mRNA and protein expression. The decrease observed in Cx43 mRNA may not reflect significant alterations in islet function since this connexin protein isoform is not present in β cells (Theis et al. 2004), which is in agreement with our results demonstrating no detectable level of this protein in pancreatic islets. The unaltered expression of Glut2 mRNA and protein in pancreatic islets from DEX rats, which was observed in the current study, is in contrast to previous data that demonstrate increased Glut2 mRNA expression in pancreatic islets from DEX nondiabetic rats (Ogawa et al. 1992). This discrepancy could be due to the higher dose and longer duration of DEX administration chosen by these authors. Thus, although no immunolocalization of Glut2 protein at β-cell membrane was carried out, this protein probably does not contribute to the islet sensitization observed in this model.

In summary, our study demonstrates the expression of Cx36 and Cx43 at mRNA and protein levels in an experimental model of insulin resistance induced by glucocorticoid DEX. This (patho)physiological condition probably results in a modestly lowered set point of islets, thus leading to the secretion of more insulin at stimulatory glucose levels and, as such, is an adaptive mechanism to counteract insulin resistance and maintain blood glucose under control (Weir et al. 2001). Based on the hypothesis that Cx36 channels provide a direct contribution to islet function, we suggest that this increase may participate in the adaptive response of islets in this model.

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ARTIGO 2

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Dexamethasone treatment in vivo counteracts the functional pancreatic islet alterations caused by malnourishment in rats

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Abstract

The effects of dexamethasone (Dex) on the metabolic parameters, peripheral insulin, and glucose sensitivity in vivo as well as on islet function ex vivo of rats submitted to low-protein diet were analyzed. Dexamethasone (1.0 mg/kg body weight) was administered intraperitoneally daily to adult Wistar rats fed on a normal-protein diet or low-protein diet (LPD) for 5 days, whereas control rats fed on a normal-protein diet or low-protein diet (LP) received saline alone. At the end of the experimental period, LP rats showed a significant reduction in serum insulin, total serum protein, and serum albumin levels compared with rats fed on a normal-protein diet ($P < .05$). All these parameters tended to be normalized in LPD rats ($P < .05$); furthermore, these rats exhibited increased serum glucose and nonesterified fatty acid levels compared with LP rats ($P < .05$). Rats submitted to the low-protein diet demonstrated normal peripheral glucose sensitivity and improved peripheral insulin sensitivity, which was reversed by Dex treatment. A reduced area of islets from LP rats was partially recovered in LPD rats ($P < .05$). At 16.7 mmol/L glucose, insulin secretion from LPD islets was also partially recovered and was significantly higher than that from LP islets ($P < .05$). In conclusion, induction of insulin resistance by Dex treatment reverses most of the metabolic alterations in rats submitted to a low-protein diet. In addition, several islet functions were also improved by Dex, confirming the plasticity of pancreatic islets in adverse conditions.

1. Introduction

The impairment of pancreatic β-cell function is the main event that leads to the development of type 2 diabetes mellitus [1]. However, before the onset of diabetes, several morphologic and physiological adaptations of the endocrine pancreas take place to maintain glucose homeostasis. These adaptations are also observed in some specific periods of life such as pregnancy, obesity, aging, and malnutrition [2-6].

Rats submitted to low-protein diet (LP) exhibit reduced body weight, higher hepatic glycogen content, normoglycemia, hypoalbuminemia, and lower plasma insulin levels [7]. The LP rat islets show impaired glucose-stimulated insulin secretion, lack of the typical secretory biphasic pattern in response to a glucose challenge, and reduction in glucose sensitivity [5,6]. Morphologic alterations such as a reduction in total pancreatic islet number are also present in this experimental model [8]. However, to compensate for the failure of pancreatic islet function, the peripheral sensitivity to insulin is increased in LP rats [9].

The endocrine adaptations to malnourishment seem to be the opposite of those observed in animals where the insulin resistance is induced by treatment with glucocorticoids. It is well known that dexamethasone (Dex) treatment impairs insulin action in peripheral tissues (ie, muscles, fat, and liver), leading to insulin resistance. Rats submitted to Dex treatment in vivo exhibit increased plasma insulin levels and marginal hyperglycemia [4,10]. In contrast to observations in islets from malnourished rats, islets from insulin-resistant rats show increased total insulin content, increased glucose-stimulated insulin secretion, and higher sensitivity to glucose based on reduced EC_{50} values. Hypertrophy of pancreatic islets is the main morphologic adaptation provoked by insulin resistance [11].

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Table 1
Composition of control (17% protein) and low-protein (6% protein) diets (Reeves et al 1993)

Ingredient	Control (17% protein)	Low protein (6% protein)
	g/kg	
Casein (84% protein)	202.0	71.5
Cornstarch	397.0	480.0
Dextrinized cornstarch	130.5	159.0
Sucrose	100.0	121.0
Soybean oil	70.0	70.0
Fiber	50.0	50.0
Mineral mix (AIN-93G)	35.0	35.0
Vitamin mix (AIN-93G)	10.0	10.0
L-Cystine	3.0	1.0
Choline chlorhydrate	2.5	2.5

The pancreatic islets exhibit morphofunctional plasticity, depending on the environment in which they evolve. However, there are no studies concerning the effects of induced insulin resistance (eg, by Dex) in an animal model that exhibits increased insulin sensitivity, such as LP rats. Here, we studied the effects of Dex treatment on metabolic adaptation of LP rats, as well as the morphophysiological alterations in their islets.

2. Materials and methods

2.1. Materials

Dexamethasone phosphate (Decadron) was from Aché (Campinas, SP, Brazil). Sodium pentobarbital (3% Hypnol) was from Cristália (Itapira, SP, Brazil). Human recombinant insulin (Biohulin N) was from Biobrás (Montes Claros, MG, Brazil). Enzymatic colorimetric assay for the quantification of nonesterified fatty acids (NEFAs) was from Wako Chemicals (Richmond, VA). Total serum protein and serum albumin detection kits were from In Vitro Diagnostica (DI, MG, Brazil). Dextrose, NaCl, KCl, $CaCl_2$, $MgCl_2$, $NaHCO_3$, KOH, and Na_2SO_4 were from Mallinckrodt Baker (Paris, France). Collagenase, HEPES, albumin, activated charcoal, and dextran were from Sigma (St Louis, MO). Ethanol, methanol, chloroform, and phenol were from Synth (Diadema, SP, Brazil).

2.2. Animals and diet

Male Wistar rats (21 days old) from the University of Campinas Animal Breeding Center were kept at 24°C on a 12-hour light/dark cycle. The rats were randomly assigned into 2 groups and fed an isocaloric diet containing 6% (LP) or 17% (control diet, NP) protein for 8 weeks. The composition and difference between the 2 isocaloric diets are described in Table 1. During the experimental period, rats had access to food and water ad lib. The institutional São Paulo State University Committee for Ethics in Animal Experimentation approved the experiments with rats.

2.3. Dexamethasone treatment

After 8 weeks of treatment, malnourished and control rats were distributed into 4 groups with 40 animals each (NP, normal-protein diet with Dex [NPD], LP, and low-protein diet with Dex [LPD]). Rats from these groups received daily intraperitoneal (IP) injections of Dex (1 mg/kg body weight, dissolved in saline) between 7:30 AM and 8:30 AM for 5 consecutive days (NPD and LPD) or saline alone (NP and LP).

2.4. Metabolic, hormonal, and biochemical measurements

On the day after the last Dex administration, fasted (12-14 hours) rats were weighed; and glucose levels were measured with a glucometer ("one touch" Johnson & Johnson, Milpitas, CA) in samples collected from the tail. The rats were then killed (by exposure to CO_2 followed by decapitation), and the blood was collected. Serum insulin levels were measured by radioimmunoassay (RIA) using rabbit anti–rat insulin antibody and rat insulin as standard. The NEFA was determined by enzyme-linked immmunosorbent assay according to the manufacturer's instructions. Total serum protein and serum albumin were quantified by spectrophotometer according to the manufacturer's instructions.

2.5. Liver glycogen measurements

Hepatic glycogen content was measured as previously described [12], but with some modifications. Briefly, the liver samples (300 to 500 mg) were transferred to test tubes containing 30% KOH (wt/vol) and boiled for 1 hour until complete homogenization. The Na_2SO_4 was then added, and the glycogen was precipitated with ethanol. The samples were centrifuged at 800*g* for 10 minutes, the supernatants were discarded, and the glycogen was dissolved in hot distilled water. Ethanol was added; and the pellets, obtained after a second centrifugation, were dissolved in distilled water in a final volume of 25 mL. Glycogen content was measured by treating a fixed volume of sample with phenol reagent and H_2SO_4. Absorbance was

Table 2
Body weight, serum protein, albumin, glucose, insulin, NEFA, and liver glycogen of NP, NPD, LP, and LPD rats

Parameters	NP	NPD	LP	LPD
Body weight (g)	409 ± 13	360 ± 12 [a]	282 ± 4 [a]	254 ± 10 [b,c]
Protein (g/dL)	6.3 ± 0.15	9.1 ± 0.14 [a]	4.9 ± 0.16 [a]	7.8 ± 0.09 [b,c]
Albumin (g/dL)	3.5 ± 0.02	4.0 ± 0.09 [a]	3.2 ± 0.09 [a]	4.3 ± 0.09 [b]
Glucose (mg/dL)	67.3 ± 4.5	135.3 ± 10.0 [a]	67.6 ± 5.9	131.3 ± 18.3 [b]
Insulin (ng/mL)	0.45 ± 0.02	1.76 ± 0.06 [a]	0.23 ± 0.02 [a]	1.70 ± 0.03 [b,c]
NEFA (mmol/L)	1.05 ± 0.15	2.04 ± 0.13 [a]	0.73 ± 0.03	1.60 ± 0.37 [b]
Liver glycogen (mg/100 mg tissue)	4.26 ± 0.3	6.68 ± 0.4 [a]	8.79 ± 0.6 [a]	9.65 ± 0.6 [c]

Values are the means ± SEM. n = 10, $P < .05$.
[a] Significantly different vs NP.
[b] Significantly different vs LP.
[c] Significantly different vs NPD.

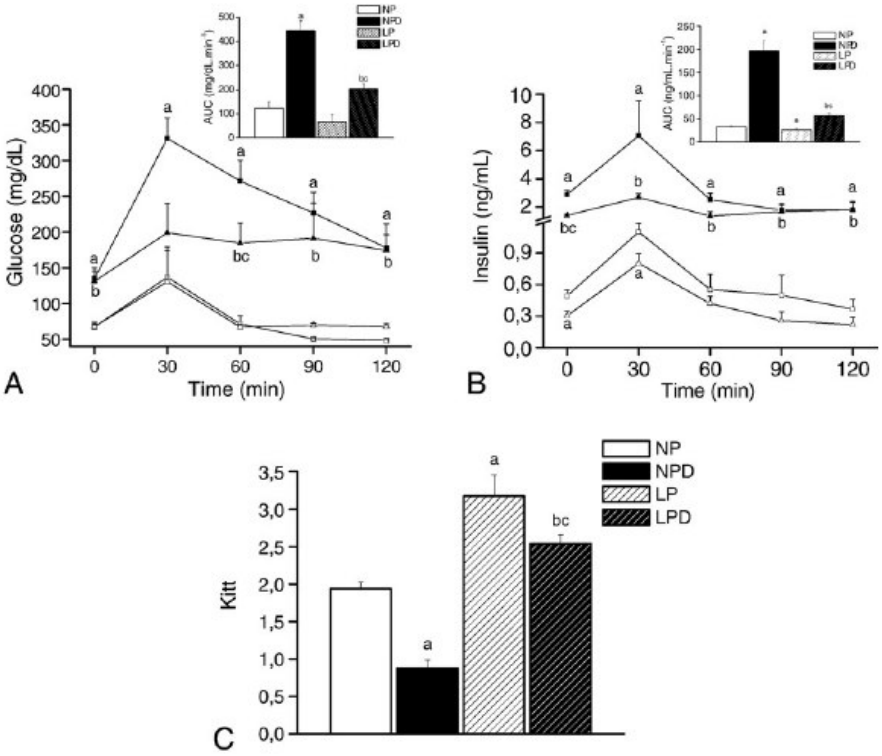

Fig. 1. Glucose intolerance in LPD rats. A, Glycemic profile obtained by ipGTT experiments in NP (o), NPD (n), LP (r), and LPD (p). Even after 120 minutes of glucose load, LPD rats showed elevation of blood glucose levels compared with LP. Note increment of area under the curve (AUC) data from LPD group compared with LP group (inset). B, Insulinemia obtained during ipGTT experiments. Blood insulin levels from LPD rats continue to be higher than those of LP rats. C, Glucose disappearance rate, measured through the blood glucose levels during the IP insulin tolerance test in NP, NPD, LP, and LPD rats. The inset depicts the increment in AUC data for LPD rats compared with LP rats. Data are means ± SEM. Significantly different [a]vs NP, [b]vs LP, and [c]vs NPD. $P < .05$, n = 10. Analysis of variance with Tukey post test.

then read at 490 nm with a spectrophotometer (Spectronic 20, Genesis, Rochester, NY).

2.6. IP glucose tolerance test

On the day after the last Dex administration, fasted (12-14 hours) rats were anaesthetized with sodium pentobarbital (3% Hypnol, 1 mL/kg body weight). After verifying the absence of corneal and pedal reflexes, unchallenged samples (time 0) were obtained from the rats' tails. Immediately, 50% glucose (2 g/kg body weight, IP) was administered; and blood samples were collected at 30, 60, 90, and 120 minutes from the tail tip for determination of glucose and insulin concentrations.

2.7. IP insulin tolerance test

Fed rats were anaesthetized as described above. A sample of blood was collected from the tail tip for glucose measurement at time 0. Human recombinant insulin, equivalent to 2 U/kg body weight, was then injected IP. Further samples were collected at 5, 10, 15, 20, 25, and 30 minutes for blood glucose measurement. The constant rate for glucose disappearance (Kitt) was calculated from the slope of the regression line obtained with log-transformed glucose values between 0 and 30 minutes after insulin administration.

2.8. Isolation of islets and static and dynamic secretion protocols

Islets were handpicked after collagenase digestion of the pancreas, following the technique previously described [13]. For static incubation, groups of 5 islets were first incubated for 1 hour at 37°C in 1 mL of Krebs-bicarbonate buffer solution of the following composition (in millimoles per liter): 115, NaCl; 5, KCl; 2.56, $CaCl_2$; 1, $MgCl_2$; 24, $NaHCO_3$; 15, HEPES; and 5.6, glucose, supplemented with 0.5% bovine serum albumin and equilibrated with a mixture of 95% O_2 and 5% CO_2 (pH 7.4). The medium was then replaced by another 1 mL of fresh buffer containing different glucose concentrations, as indicated in the respective figure, and further incubated for 1 hour. At the end of the incubation, the samples were stored at –20°C for subsequent measurement of insulin content by RIA. For analysis of dynamic insulin secretion, 20 freshly isolated islets were transferred to perfusion chambers and perifused with Krebs-bicarbonate

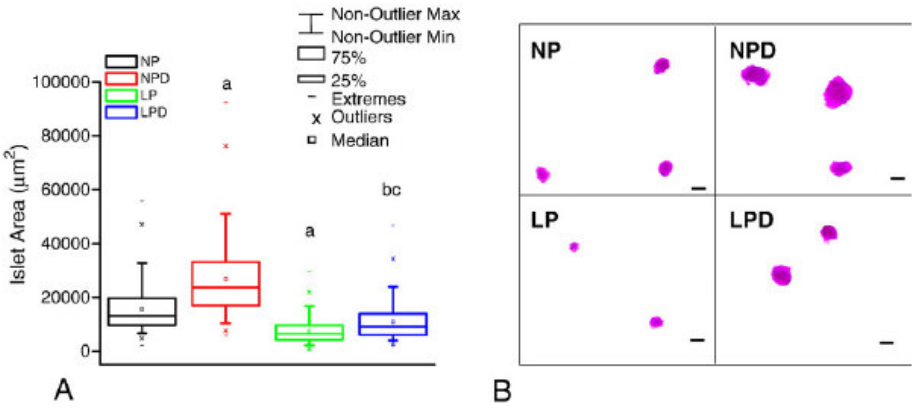

Fig. 2. Adaptive hypotrophy and hypertrophy of pancreatic islet. A, Box plot graphic representation of islet area values. Each plot is the mean of at least 700 islets per group. B, Representative images obtained from a charge-coupled device camera. Significantly different [a]vs NP, [b]vs LP, and [c]vs NPD. $P < .001$. Analysis of variance with Tukey post test; ×100 magnification. The bar corresponds to 100 μm.

buffer solution at a flow rate of 1 mL/min for 100 minutes. The effluent was collected every 2 minutes into tubes that were stored at –20°C for insulin RIA. Perifusion consisted of 3 consecutive periods: 50 minutes with 2.8 mmol/L glucose, 30 minutes with 16.7 mmol/L glucose, and finally 20 minutes with 2.8 mmol/L glucose. Collection of the samples started from 30 minutes of perifusion.

2.9. Histomorphometric analysis

To determine the area, pools of 700 islets from each group were submitted to Feulgen DNA method "en bloc." The islet images were then registered by a charge-coupled device camera, and area (in square micrometers) values were automatically obtained by the Image-Pro-Plus Media, Cybernetics program (Bethesda, MD), coupled to a BX-60 Olympus photomicroscope (Tokyo, Japan).

2.10. Statistical analysis

All numerical results are expressed as the means ± SEM of the indicated number of experiments. Analysis of variance (1-way) for unpaired groups, followed by Tukey post test, was used for multiple comparisons of parametric data. The significance level adopted was $P < .05$.

3. Results

3.1. Characteristics of the rats

The LP rats showed a significant reduction in body weight, total serum protein, albumin, and insulin serum levels compared with NP rats (n = 10, $P < .05$). The liver glycogen content of LP rats increased significantly, and serum glucose levels were similar to those of NP rats (n = 10, $P < .05$) (Table 2). After Dex treatment, however, total serum protein, serum albumin, and NEFA levels of LPD rats increased significantly compared with LP rats (n = 10, $P < .05$). Blood glucose and insulin in LPD rats were significantly higher compared with LP rats (n = 10, $P < .05$). The liver glycogen content was similar between LPD and LP rats (Table 2). The data for NPD rats were similar to those previously reported by others [14-16].

3.2. LPD rats exhibit decreased glucose and insulin sensitivity

The mean glucose blood levels during the intraperitoneal glucose tolerance test (ipGTT), except for 30 minutes, in LPD rats were significantly higher than those in LP rats (n =10, $P < .05$), but similar to those found in NPD rats. No differences were observed between LP and NP rats (Fig. 1A). Blood insulin levels were significantly increased at all times in LPD rats compared with LP rats, suggesting glucose intolerance in this group (n = 10, $P < .05$) (Fig. 1). During the IP insulin tolerance test, LP rats were more sensitive to insulin than NP rats, as judged by the Kitt values (Fig. 1C, $P < .05$). Although LPD rats showed a higher sensitivity to insulin than NP and NPD groups, this sensitivity was significantly lower than that of LP rats ($P < .05$). The Kitt values were 0.45-fold, 1.67-fold, and 1.31-fold altered compared with the NP value for the NPD, LP, and LPD groups, respectively (Fig. 1C; n = 10; $P < .05$ for LP vs NP, NPD vs NP, and LPD vs LP groups).

3.3. Morphologic islet adaptation in LP and LPD rats

The LP islets showed a marked hypotrophy compared with NP islets. However, Dex treatment caused a significant increase in islet area in NPD and LPD rats compared with NP and LP rats, respectively (Fig. 2). The islet area values were 15700 ± 320, 26800 ± 530, 7500 ± 180, and 10900 ± 250 μm^2 for the NP, NPD, LP, and LPD groups, respectively (n = 700 islets, $P < .001$). This may reflect an adaptation imposed by the increased sensitivity and decreased sensitivity to insulin action at the periphery in LP and LPD animals, respectively.

3.4. Glucose-stimulated insulin secretion is increased in LPD rats

As depicted in Fig. 3, after normalization by the total islet insulin content, at a subthreshold glucose concentration (2.8 mmol/L), insulin secretion was significantly decreased in LP and LPD (0.57-fold and 0.5-fold compared with NP and LP, respectively). At 16.7 mmol/L glucose, the insulin secretion was significantly decreased in LP islets (0.17-fold compared with NP islets, n = 12, $P < .05$), whereas the insulin secretion was significantly increased in NPD and LPD islets (2.4-fold and 1.7-fold higher compared with NP and LP islets, respectively; n = 12; $P < .05$) (Fig. 3A). The dose-response curve to glucose (2.8-22 mmol/L) was significantly shifted to the right in LP compared with NP islets. Nevertheless, the dose-response curve to glucose was shifted to the left in islets derived from the NPD and LPD groups compared with their respective controls (Fig. 3B). The EC_{50} values were 9.08 ± 0.10, 6.84 ± 0.32, 9.78 ± 0.19, and 7.11 ± 0.46 mmol/L for NP, NPD, LP, and LPD, respectively ($P < .05$ for LP vs NP, NPD vs NP, and LPD vs LP).

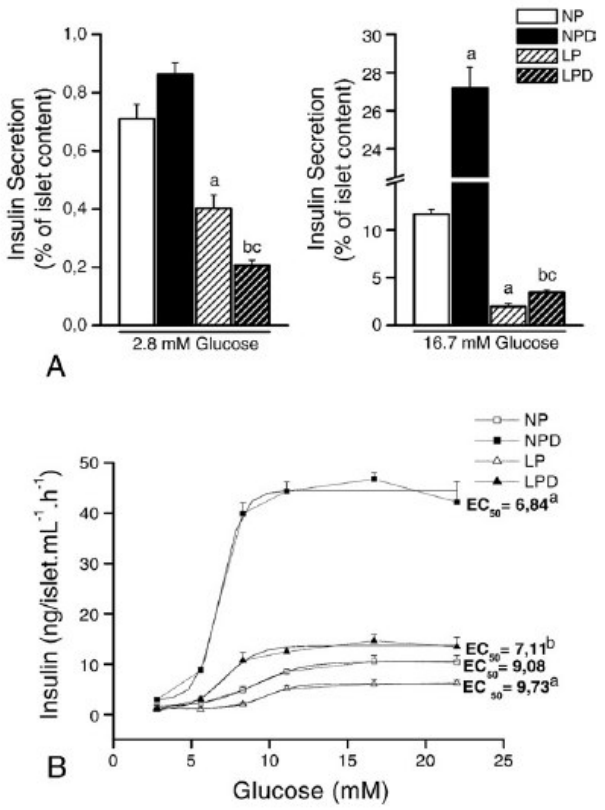

Fig. 3. The LPD islets show increased glucose-induced insulin release. Insulin release from islets isolated from NP, NPD, LP, and LPD rats. A, At a concentration of 2.8 mmol/L, glucose insulin secretion was not different between NP vs LP and LP vs LPD. At a stimulatory glucose concentration (16.7 mmol/L), the insulin secretion in LP islets was diminished compared with NP islets; however, in LPD islets, the insulin secretion was 150% higher than that of LP islets. The absolute values for insulin secretion at 2.8 mmol/L glucose were 0.5 ± 0.04, 0.9 ± 0.04, 0.2 ± 0.02, and 0.2 ± 0.01 ng per islet per milliliter per hour for NP, NPD, LP, and LPD, respectively ($P < .05$ for LP vs NP and NPD vs NP). At 16.7 mmol/L glucose, insulin secretion was 8.4 ± 0.3, 29.3 ± 1.2, 1.2 ± 0.2, and 2.9 ± 0.6 ng per islet per milliliter per hour for NP, NPD, LP, and LPD, respectively ($P < .05$ for LP vs NP and NPD vs NP). B, Dose-response curve to glucose (2.8-22 mmol/L) obtained from insulin release by static incubation of the islets. The EC_{50} value was significantly shifted to the right in LP islets compared with NP islets and significantly shifted to the left in LPD islets compared with LP islets. Data are means ± SEM. Significantly different [a]vs NP, [b]vs LP, and [c]vs NPD. $P < .05$, n = 12. Analysis of variance with Tukey post test.

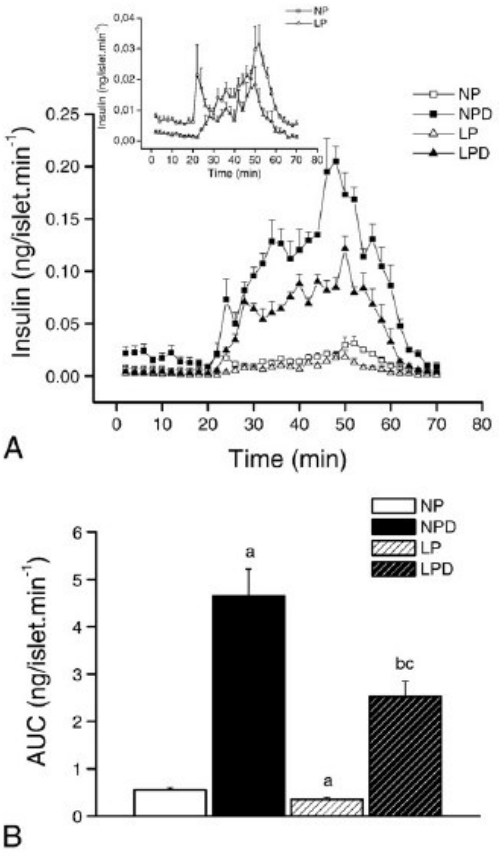

Fig. 4. Perifused LPD islets show altered insulin-output pattern. Glucose-stimulated insulin secretion in isolated perfused islets of NP, NPD, LP, and LPD rats. The typical biphasic insulin pattern was observed in NP islets (inset). The LP islets showed no characteristic first phase and a second phase of insulin release that was lower than that in NP islets. The LPD islets showed both phases of insulin release with a marked and sustained second phase compared with LP islets. The inset shows the insulin secretion from NP and LP islets. B, The AUC data revealed an increased insulin response to glucose in NPD and LPD. A, Each point represents the mean ± SEM of 4 different experiments. B, Data are means ± SEM. Significantly different [a]vs NP, [b]vs LP, and [c]vs NPD. $P < .05$, n = 12. Analysis of variance with Tukey post test.

3.5. Kinetics of glucose-induced insulin secretion in LPD islets

In the presence of nonstimulatory glucose concentrations (minutes 0-20), the islets from the NPD and LPD groups released more insulin than their respective controls (Fig. 4).

A typical biphasic pattern of insulin secretion was observed in NP islets after the introduction of 16.7 mmol/L glucose (minutes 20-50) (inset). The insulin secretion in LP islets did not show a characteristic first-phase release; but despite a lower release than NP islets, the second phase exhibited a sustained increase in insulin release. The LPD islets showed a biphasic response to glucose, with a first peak followed by a sustained and progressive increased second phase. Insulin secretion in both phases was significantly higher in LPD than LP islets. As expected, NPD islets secreted significantly more insulin during stimulation with 16.7 mmol/L glucose than NP islets. Finally, the insulin secretion in all groups of islets returned to basal values when the glucose concentration was reduced to 2.8 mmol/L glucose (minutes 50-70).

4. Discussion

In this study, several physiological parameters were assessed in adult Wistar rats submitted to a low-protein diet (28 to 90 days of life) and turned resistant to insulin by the administration of Dex. Morphometric adaptations, provoked by these treatments, were also investigated as well as alterations in insulin secretion in isolated pancreatic islets.

Rats fed on a low-protein diet (LP) had similar characteristics to those observed in experimental malnourishment models, such as a reduction in body weight and serum protein levels and an increase in hepatic glycogen content [5,7]. These biochemical parameters were modified by the treatment of NP and LP rats with Dex and are in agreement with data reported in the literature concerning glucocorticoid-induced insulin resistance, that is, body weight reduction [17-19] and increase in plasma protein levels [10,15,16], NEFAs [14,20,21], and hepatic glucose output [10,22]. The body weight reduction is usually accompanied by muscle atrophy [16,17,19], probably due to an increase in cathepsin L expression, one of the main mediators of muscle proteolysis [17]. The proteolysis caused by glucocorticoid treatment [23] might justify the elevated serum protein in our NPD and LPD rats. Glucocorticoids also stimulate lipolysis and potentiate the lipolytic effect of other hormones, leading to an increase in serum NEFA concentrations [24], in accordance with the increased serum NEFA concentration in LPD rats. Higher NEFA plasma levels could increase hepatic glycogen synthesis because the increase in NEFA may suppress hepatic glycolysis and favor hepatic glycogen synthesis [25].

The data regarding islet area reported herein are also in agreement with previous data, suggesting a reduction in islet area in malnourished animal models [8] and an increase in islet area in glucocorticoid-treated animals [10,11,26,27]. The islet hypotrophy is probably an adaptation in the protein-restricted rats to a lower body weight associated with an increased sensitivity to insulin in periphery tissues. The effects on islet morphology imposed by malnutrition in LPD rats were counteracted by an adaptive response to the development of peripheral insulin resistance induced by the Dex treatment. This increase in islet size seems to be a result of increased β-cell number and/or size [28].

The postabsorptive insulin levels are another interesting finding of our study. The LP rats showed a significant reduction in insulin serum levels; and according to previous results, this may be explained by a decreased β-cell mass associated with functional alterations such as impairment of nutrient metabolism and calcium uptake [5,6,8]. Moreover, these reduced insulin levels could reflect a response to the increased sensitivity to glucose peripherally, which, in turn, would demand less insulin to maintain glucose homeostasis. Despite low insulin levels, LP rats showed normal blood glucose and normal glucose tolerance together with an improvement in the peripheral insulin sensitivity as judged by the Kitt, in agreement with a previous study [29]. The improvement in insulin sensitivity might be a consequence of an increase in the phosphorylation of insulin receptor (IR) and IR substrate 1, favoring a greater association of IR substrate 1 with phosphatidylinositol-3-kinase [9]. In contrast, LPD rats exhibited increased blood glucose and insulin levels associated with decreased peripheral insulin and glucose sensitivity. Increased insulin secretion is an adaptive response of β-cells, imposed by the Dex-induced peripheral insulin resistance [27] and mediated by concomitant elevation of blood glucose levels [30]. The hyperglycemic effect of glucocorticoids is explained, at least in part, by their gluconeogenic action on hepatic tissue. In addition, glucocorticoids induce muscle and fat tissue insulin resistance, which contributes to the aggravation of hyperglycemia [31,32].

We also demonstrated alterations in lipid metabolism, particularly increases in NEFA levels in NPD and LPD groups. Evidence exists to suggest that glucocorticoids may increase serum NEFA levels by the activation of hormone-sensitive lipase [33]. The increases in serum insulin concentrations observed in NPD and LPD rats, however, were not sufficient to avoid hypertriglyceridemia and increased NEFA concentrations in this group. It has been proposed [34] that the increase in plasma NEFA concentrations, while contributing to the induction or aggravation of peripheral insulin resistance, could, in parallel, mediate insulin hypersecretion either directly [35,36] or by favoring triglyceride synthesis in β-cells and subsequent generation of lipid signaling molecules through lipolysis [37]. Elevated serum NEFA levels have been implicated in the pathogenesis of glucocorticoid-induced peripheral insulin resistance [14,20,21].

We also studied insulin release in isolated islets ex vivo in these rats. In agreement with a previous study [5], LP islets showed an impairment of glucose-induced insulin release, probably as a consequence of a reduction in islet number per pancreas, as well as a reduction in the quantity, size, and volume of β-cells [8]. In addition, the lower ability of glucose to induce Ca^{2+} uptake and/or to reduce Ca^{2+} efflux from β-cells could play an important role in this process [5].

However, LPD islets showed augmented glucose-induced insulin secretion in stimulatory (16.7 mmol/L) glucose concentrations associated with lower EC_{50} values for glucose. Thus, β-cells from LPD rats are more responsive to glucose. Controversies exist concerning the secretion of insulin from perfused pancreas and isolated islets of rodents after in vivo glucocorticoid treatment. Enhanced [38,39] or unaltered [40] insulin secretion has been described in both preparations. These heterogeneous results are linked to the countless protocols used (strain, dose, time, glucose concentration, etc). Our results suggest that the increase in insulin secretion observed in the LPD group, in response to suprathreshold glucose concentrations, implies some degree of islet adaptation (ie, decrease in EC_{50} values for glucose), which could be a reflection of the impairment of insulin action at the periphery.

Finally, we demonstrated that LP rats exhibit an improvement in insulin sensitivity, a normal glucose tolerance, a diminished islet area, and an impairment of islet function, reflecting an adaptation imposed by the protein deficiency in the diet. However, when insulin resistance is induced by Dex treatment in LP rats, several adaptations occur at the periphery as well as in the islets to counteract these common features induced by the malnourishment. The classic metabolic and islet alterations observed in rodents submitted to Dex-induced insulin resistance include the decrease of insulin and glucose sensitivity, islet hypertrophy, and enhanced glucose-stimulated insulin secretion. All of these parameters are also observed in LPD rats even after well-established alterations imposed by the protein restriction in the diet. Thus, Dex reverts the main features related to metabolism and islet function caused by protein restriction in the diet. These observations may exemplify and reinforce the wide plasticity of pancreatic β-cells in adverse conditions. Understanding these adaptations is of relevance because they may lead us to classify the degrees of these pathophysiological conditions, providing directions for future studies.

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ARTIGO 3

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EFEITOS DA ADMINISTRAÇÃO DE DEXAMETASONA *IN VIVO* SOBRE GLICEMIA, INSULINEMIA E SUBSTRATOS CIRCULANTES SÃO DEPENDENTES DO TEMPO DE TRATAMENTO

EFFECTS OF DEXAMETHASONE ADMINISTRATION IN VIVO *ON GLYCAEMIA, INSULINAEMIA AND CIRCULATING SUBSTRATES ARE DEPENDENTS OF TIME OF TREATMENT*

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RESUMO: Terapias a base de glicocorticóides estão freqüentemente associadas a alteração da sensibilidade à insulina. No presente trabalho avaliamos alguns parâmetros metabólicos como glicose, insulina, proteínas e colesterol plasmáticos em ratos tratados com dexametasona (DEX) (1mg/kg, peso corpóreo, *ip.*) por diferentes períodos de tempo (24h, 72h e 120h). Os ratos tratados com dexametasona apresentaram resistência periférica à insulina após 24h de administração da droga como indicam os valores de insulina plasmática de jejum (1,3 *vs.* 6,8 ng/ml para ratos controle [CTL] e DEX, respectivamente) e do índice HOMA. Resistência periférica à insulina adicional ocorre até o final do tratamento nos ratos DEX. A glicemia permanece moderadamente elevada até o período de 72h. Entretanto, observa-se marcante hiperglicemia após 120h (79 *vs.* 160 mg/dl para ratos CTL e DEX, respectivamente). Aumento significativo dos níveis de proteínas totais e albuminas plasmáticas ocorre a partir de 72h de tratamento e de colesterol total a partir de 120h. Glicogênio e gordura hepáticos aumentam de maneira tempo-dependente nos ratos DEX. Correlação negativa foi observada entre os valores de insulinemia de jejum e peso nos grupos tratados com dexametasona ($r > 0,95$). Portanto, administração de dexametasona, 1mg/kg, induz resistência periférica à insulina de maneira tempo-dependente a partir de 24h e aumento dos níveis circulantes de glicose e proteínas plasmáticos após 72h de tratamento.

PALAVRAS-CHAVE: Carboidratos. Glicocorticóides. Gorduras. Proteínas.Ratos. Tempo-dependente.

INTRODUÇÃO

Os glicocorticóides são hormônios sintetizados e secretados pelo córtex adrenal. Esta classe de hormônios exerce inúmeras ações fisiológicas no organismo quando disponíveis em concentrações normais. Os glicocorticóides atuam normalmente sobre o metabolismo dos carboidratos, proteínas e gorduras e são secretados em maiores quantidades em situações traumáticas e estressantes. Entretanto, quando as concentrações plasmáticas dos glicocorticóides excedem o normal, podem induzir distúrbios metabólicos. Portadores da Síndrome de Cushing (NOSADINI et al., 1983), pacientes submetidos à terapia de corticosteróides com fins anti-inflamatórios bem como aqueles submetidos a transplante de órgãos com subseqüente medicação com imunossupressores (MORA, 2005) são exemplos comuns em que se observam aumento das concentrações plasmáticas de glicocorticóides. Nestes casos, os glicocorticóides apresentam-se como hormônios diabetogênicos, pois promovem aumento da produção hepática de glicose e diminuição da captação periférica deste açúcar em tecidos como muscular, hepático e adiposo (SAAD, 1994).

Diversos autores têm demonstrado alterações metabólicas em modelos experimentais com roedores *in vivo* por administração de glicocorticóide. Perda de massa muscular associada à hipertrofia do fígado (SAVARY et al., 1998) redução do peso corpóreo em ratos (CALDEFIE-CHAZET et al., 2001, SAVARY et al., 2001) alteração do perfil protéico (RUZZIN; WAGMNAN; JENSEN, 2005) e do perfil lipídico (NOVELLI et al., 1999; BARBERA et al., 2001; HOLNESS et al., 2005) são características comuns após tratamento com dexametasona. Aumento dos níveis plasmáticos de glicose e de insulina (NOVELLI et al., 1999; BARBERA et al., 2001) também são observados.

Os efeitos dos glicocorticóides sobre o metabolismo podem ser particularmente dependentes da dose e do tempo de administração. No melhor de nosso conhecimento nenhum trabalho demonstrando os efeitos da dexametasona sobre parâmetros metabólicos em diferentes períodos de tratamento *in vivo* foi descrito em ratos. Assim, o

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presente trabalho propõe-se a investigar alguns parâmetros metabólicos como glicose, insulina, proteínas e colesterol plasmáticos, bem como glicogênio e gordura hepáticos em ratos tratados com dexametasona (1mg/kg, peso corpóreo, *ip.*) em diferentes períodos (24h, 72h e 120h) com o intuito de observar o curso em que as alterações ocorrem bem como identificar os primeiros parâmetros alterados.

MATERIAIS E MÉTODOS

Materiais

Fosfato de dexametasona e reagentes utilizados para dosagem de insulina por radioimunoensaio (RIA) foram adquiridos de Sigma (St. Louis, MO, E.U.A). Kits enzimáticos para quantificação de proteína total e albumina plasmática foram adquiridos de In Vitro Diagnostica (DI, MG, Brasil). Kit enzimático para dosagem de colesterol total plasmático foi obtido de In vitro-Human (Wiesbaden, Germany). Os sais utilizados para dosagem do glicogênio hepático foram adquiridos de Mallinckrodt Baker, Inc. (Paris, Kentucky, França). Etanol, metanol, clorofórmio e fenol foram adquiridos de Synth (Diadema, SP, Brasil).

Animais e tratamento com dexametasona

Foram utilizados ratos Wistar machos com 90 dias de idade, provenientes do Biotério Central da UNESP, localizado no Câmpus de Botucatu, SP. Os ratos foram mantidos sob temperatura controlada (24 ± 2 °C) em ciclo de iluminação claro-escuro (12 horas), com livre acesso à comida e água. Os animais receberam injeção intraperitoneal diária (entre 7:30 e 8:30 h) de solução salina, 1 ml/Kg peso corpóreo (grupo CTL) ou fosfato de dexametasona, 1 mg/Kg peso corpóreo (grupo DEX) e divididos em subgrupos de acordo com os tratamentos: uma única injeção (grupos CTL24h e DEX24), injeção diária por três dias consecutivos (grupos CTL72h e DEX72h) ou injeção diária por cinco dias consecutivos (grupos CTL120h e DEX120h). Após jejum de 12h os animais foram sacrificados por exposição ao CO_2, seguido por decapitação no dia seguinte à última administração para cada grupo. Os procedimentos experimentais foram aprovados pelo Comitê de Ética em pesquisa da Faculdade de Ciências, Bauru, SP.

Parâmetros Metabólicos

Crescimento e consumo alimentar: os ratos foram mantidos em gaiolas metabólicas e o peso corpóreo e o consumo alimentar foi acompanhado durante os dias de tratamento em balança eletrônica (Filizola) entre 7:30 e 8:30 h.

Glicemia: foi quantificada pelo sistema de fitas (Roche – Diagnostics) a partir de sangue retirado da cauda.

Insulinemia: imediatamente após decapitação o sangue foi coletado em tubos Falcon sem heparina. Em seguida, as amostras foram centrifugadas a 3000rpm a 4 ºC durante 15 min (eppendorf 5810R) e o sobrenadante foi armazenado a -20 °C para posterior quantificação da insulina por RIA utilizando anticorpo anti-insulina obtida em coelho e insulina de rato como padrão (SCOTT; ATWATER; ROJAS, 1981).

Proteína total, albumina e colesterol total: foram dosados de acordo com as instruções do fabricante com kits específicos através de métodos enzimáticos e mensurados por espectrofotometria (Spectronic 20 genesis).

Glicogênio Hepático: a determinação do glicogênio hepático ocorreu de acordo com (LO; RUSSEL; TAYLOR, 1970) com algumas modificações. Brevemente, amostras de fígado (300 a 500 mg) foram colocadas em tubos de ensaio contendo solução de KOH 30 % (w/v) e levados ao banho-maria fervente por 1 h até completa homogeneização. Em seguida, foi adicionado 0,2 ml de solução de Na_2SO_4 saturada e 6 ml de etanol para precipitação do glicogênio. Os tubos foram centrifugados a 2000 rpm por 10 min (eppendorf 5810R), o sobrenadante descartado e os "pellets" de glicogênio ressuspendidos em 2 ml de água destilada quente. Etanol (6ml) foi adicionado e os "pellets", obtidos após centrifugação, foram ressuspendidos com água destilada em volume final de 20 ml. O conteúdo de glicogênio foi determinado a partir do tratamento de 0,2 ml das amostras com 15 µ de solução de fenol, 0,8 ml de água destilada e 2 ml de H_2SO_4 com posterior leitura da absorbância em espectrofotômetro a 490 nm (Spectronic 20 genesis).

Gordura Hepática: aproximadamente 100 mg de fígado foram homogeneizados (Polytron PT 1200C) em 7,5ml de solução metanol/clorofórmio (1:2, v/v), mantidos em repouso "overnight" em temperatura ambiente para posterior filtração e pesagem da gordura precipitada.

Avaliação da Sensibilidade Insulínica periférica pelo Modelo de Análise Homeostática (HOMA)

Calculado a partir da fórmula: glicemia (mmo/l) x insulinemia (µŪ/ml)/22.5 (MATTHEWS; HOSKER; RUDENSKI, 1985).

ANÁLISE ESTATÍSTICA

Os resultados foram expressos como média ± erro padrão da média (EPM) do número indicado (n) de experimentos. Teste t de "student" independente foi utilizado para comparação do grupo CTL com seu respectivo grupo DEX. Análise de variância de uma via (one way - ANOVA), para grupos emparelhados e não emparelhados, seguido de *post test* Tukey foi utilizada para comparação múltipla de valores paramétricos (intra-grupos). Correlação entre varíaveis dependentes foi aplicada e foram considerados como significante os valores com r ≥ 0,9. O nível de significância adotado foi de $P < 0,05$.

RESULTADOS

Ratos tratados com dexametasona apresentam redução de massa corpórea e ingestão alimentar após 48h de tratamento

O acompanhamento do peso corpóreo e da ingestão alimentar deu-se diariamente, a fim de construir uma curva de variação ao longo do tratamento. Para isso os dados foram obtidos dos animais pertencentes aos grupos 120h, ou seja, com cinco dias de tratamento. Como mostra a figura 1A, dois dias após a administração de dexametasona nota-se redução significativa de massa corporal em relação ao grupo CTL ($n = 10$, $P < 0,05$). Reduções de 11%, 14% e 19% foram observadas nos dias 3, 4 e 5 em relação aos valores do grupo CTL, respectivamente ($n = 10$; $P < 0,05$ para todos os períodos superiores a 24h de tratamento). Redução significativa de ingestão alimentar ocorreu após dois dias de administração da droga comparado aos ratos CTL que apresentaram perfil inalterado ao longo do tratamento com salina ($n = 10$; $P < 0,05$). Interessantemente, houve recuperação da ingestão alimentar, ainda que em quantidade menor do que comparado ao grupo CTL, até o encerramento do tratamento ($P < 0,05$) (Figura 1B).

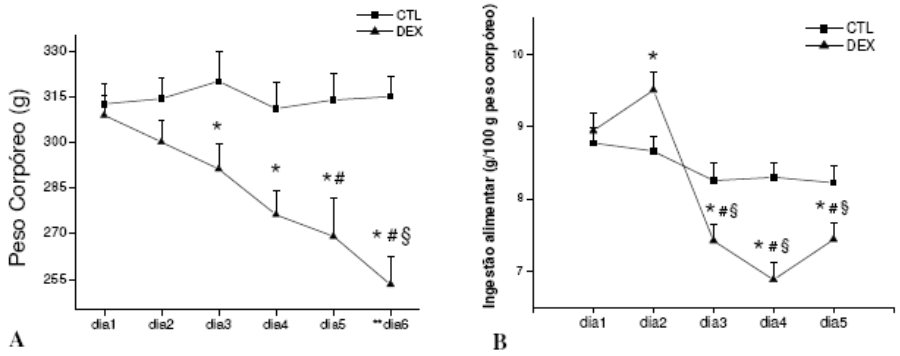

Figura 1. Crescimento e consumo alimentar durante tratamento com dexametasona em ratos. Observa-se redução de peso corporal (**A**) e ingestão alimentar (**B**) no grupo DEX. **dia da eutanásia. $n = 10$. * diferença significante *vs.* CTL, $P < 0,05$, teste "t" de Student. # *vs.* Dia 1; § *vs.* dia 2 e 3 para peso corpóreo e *vs.* dia 2 para ingestão alimentar, $P < 0,05$, ANOVA com *post test* Tukey.

Administração de dexametasona induz resistência à insulina de maneira tempo-dependente e aumento dos níveis plasmáticos de glicose, proteínas totais, albumina, colesterol e glicogênio e gordura hepáticos.

Os grupos DEX mostraram níveis de insulina de jejum significativamente maiores, de maneira tempo-dependente, quando comparados aos grupos CTL (6,8 ± 2,2 *vs.* 1,3 ± 0,35, 8,4 ± 2,2 *vs.* 1,4 ± 0,44 e 9,8 ± 2,3 *vs.* 1,6 ± 0,5 ng/ml para ratos DEX 24h, DEX 72h e DEX 120h e respectivos CTL, respectivamente; $n = 10$, $P < 0,05$) (Figura 2A). Correlação negativa foi observada entre os valores de insulinemia de jejum e peso (no dia da eutanásia) nos grupos tratados com dexametasona ($r = 0,99$).

Os níveis de glicose sanguínea também se apresentaram maiores que nos grupos CTL sendo marcante nos ratos DEX 120h (90,5 ± 5 *vs.* 76 ± 3,2, 96,8 ± 7 *vs.* 80 ± 3,8 e 160 ± 18,7 *vs.* 79 ± 4 mg/dl para ratos DEX 24h, DEX 72h e DEX 120h e respectivos CTL, respectivamente; $n = 10$, $P < 0,05$) (Figura 2B). Confirmação da instalação de resistência à insulina foi obtida pelo cálculo do índice HOMA (figura 2C).

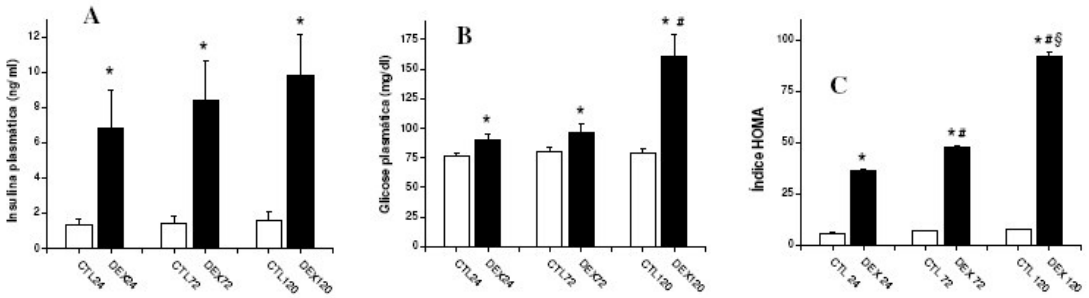

Figura 2. Insulinemia,glicemia e índice HOMA em ratos DEX e CTL. Houve aumento de maneira tempo-dependente dos níveis plasmáticos de insulina (**A**) e no índice HOMA (**C**). A glicemia é maior em todos os grupos DEX comparados aos grupos CTL (**B**). $n = 10$. *

Aumento da concentração plasmática de proteínas totais (1,2 e 1,5 vezes) e albumina (1,3 vezes) ocorreu em ratos DEX 72 e DEX 120 *versus* respectivos CTL ($n = 10$, $P < 0,05$) (Figura 3A -B). Entretanto, houve aumento significativo de colesterol total plasmático somente após 120h de tratamento (94,4 ± 5,6 e 70 ± 7 mg/dl para ratos DEX 120h *vs.*CTL, respectivamente; $P < 0,05$) (Figura 3C). Os grupos tratados com dexametasona apresentaram valores de glicogênio hepático significativamente maiores, de maneira tempo-dependente, comparado aos grupos CTL (4,3 ± 0,5 *vs.*1,2 ± 0,1, 5,1 ± 0,3 *vs.*1,4 ± 0,3 e 5,9 ± 0,1 *vs.* 1,1 ± 0,2 mg/100g tecido para ratos DEX 24h, DEX 72h e DEX 120h e respectivos CTL, respectivamente; $n = 10$, $P < 0,05$) (Figura 4A). Aumento significante do conteúdo de gordura hepática foi observado nos ratos DEX 72h e DEX 120h ($n = 10$, $P < 0,05$) e aumento marginal no grupo DEX 24h ($P > 0,05$, NS) (Figura 4B).

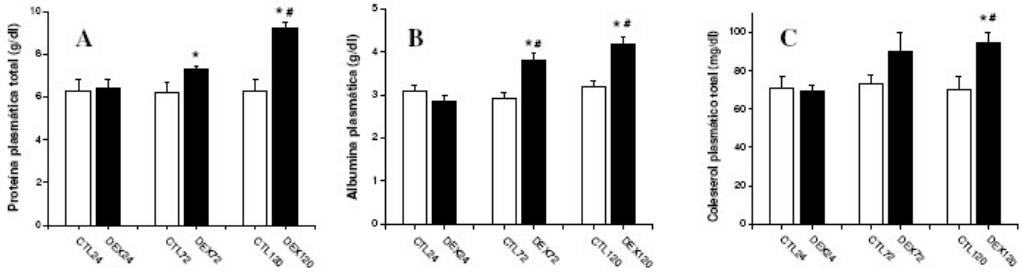

Figura 3. Proteínas totais, abumina e colesterol total plásmáticos em ratos DEX e CTL. Observa-se aumento após 72h de tratamento com dexametasona nos níveis de proteínas totais (**A**) e albumina (**B**). Aumento dos níveis de colesterol ocorreu após 120h de tratamento (**C**) nos ratos DEX comparados aos ratos CTL $n = 10$. * diferença significante *vs.* respectivo CTL, $P < 0,05$, teste "*t*" de Student. # *vs.* DEX 24h para albumina e colesterol e *vs.* DEX 24h e DEX 72h para proteínas totais. # *vs.* DEX 24h; § *vs.* DEX 24h e DEX 72h, $P < 0,05$, ANOVA com *post test* Tukey

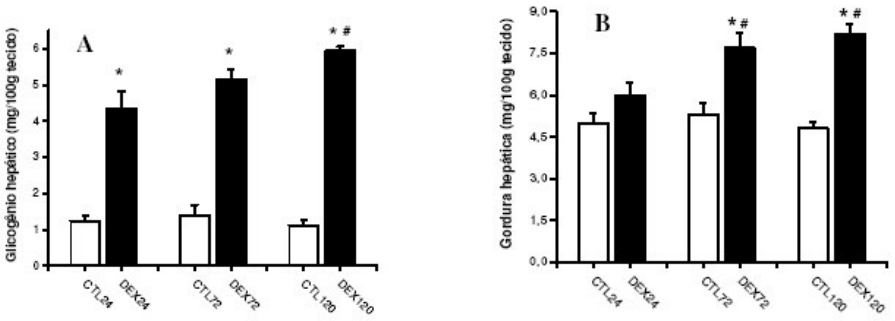

Figura 4 . Glicogênio e gordura hepáticos em ratos DEX e CTL. Houve aumento de glicogênio (**A**) e gordura (**B**) hepáticos nos ratos DEX comparados aos ratos CTL $n = 10$. * diferença significante *vs.* respectivo CTL, $P < 0,05$, teste "*t*" de Student. # *vs.* DEX 24h, $P < 0,05$, ANOVA com post test Tukey.

DISCUSSÃO

No presente trabalho acessamos parâmetros metabólicos plasmáticos em ratos tratados por 24, 72 e 120h com dexametasona (1mg/kg, ip). Alguns substratos como glicose, proteína s e lipídios geralmente apresentam-se alterados durante terapias a base de glicocorticóides (ANDREWS; WALKER, 1999) e em modelos experimentais tratados com corticosteróides (NOVELLI, et al., 1999; BARBERA et al., 2001; CESARETTI; KOHLMANN Jr., 2006). Um dos principais resultados no trabalho corrente é a observação de que aumento dos níveis circulantes de insulina plasmática é a primeira alteração manifestada após 24h de administração da droga concomitante com pequena elevação das concentrações de glicose sanguínea.

A elevação dos níveis de insulina pode ser explicada parcialmente como resposta adaptativa das células B pancreáticas a eventos periféricos como a resistência à insulina desenvolvida na presença de concentrações elevadas de glicocorticóides. Diversos autores demonstraram o efeito direto dos glicocorticóides sobre tecidos periféricos insulino-dependentes como o tecido muscular, adiposo e hepático (SAAD; FOLLI; KHAN, 1995; WEINSTEIN et al., 1998, BURÉN et al., 2002; RUZZIN; WAGMNAN; JENSEN, 2005). Ratos tratados com dexametasona (0.9 mg/kg por 2 dias) exibem captação reduzida de 2-[^{3}H]deoxiglicose em tecido muscular após estimulação por insulina e diminuição de marcador de superfície celular para o transportador de glicose tipo 4 (GLUT-4) (WEINSTEIN et al., 1998). A diminuição da sensibilidade à insulina em tecido muscular proveniente de ratos tratados por 12 dias consecutivos com dexametasona também foi constatada pela diminuição da captação de glicose neste tecido (RUZZIN; WAGMNAN; JENSEN, 2005). Adipócitos isolados de ratos cultivados na presença de dexametasona por 24h, independente da concentração de glicose presente no meio, reduzem significativamente a captação de glicose basal e induzida por insulina (BURÉN et al., 2002). No tecido muscular esquelético bem como no tecido adiposo a resistência à insulina induzida por dexametasona parece ser mediada por mecanismos pós-receptores. No primeiro, ocorre diminuição da fosforilação da proteína cinase B (PKB) e da proteína glicogênio sintase cinase-3 (GSK-3) estimuladas pela insulina (RUZZIN; WAGMNAN; JENSEN, 2005) além de falha no recrutamento de Glut4 para superfície celular (WEINSTEIN et al., 1998). No tecido adiposo foi demonstrada redução do conteúdo total de substrato do receptor de insulina-1 (IRS-1) e da PKB acompanhado por redução paralela da ativação da PKB estimulada pela insulina (BURÉN et al., 2002).

Outro dado interessante do atual trabalho é o aumento da insulinemia ao longo do tratamento com dexametasona e concomitante aumento das concentrações de glicose sanguínea, especialmente no grupo DEX 120h. A adaptação das ilhotas pancreáticas frente ao quadro de resistência periférica inclui diversas alterações funcionais e morfológicas. Tem sido amplamente demonstrado aumento da secreção de insulina tanto *in vivo,* após sobrecarga de glicose no teste de tolerância à glicose (DAVANI et al., 2004; HOLNESS et al., 2005) quanto *ex vivo* em ilhotas perfundidas ou incubadas na presença de concentrações estimulantes de

inúmeros secretagogos, em especial de glicose em roedores tratados com dexametasona (MALAISSE et al., 1967; KAWAI; KUZUYA 1977; WANG et al. 1994; NOVELLI et al. 1999). Do ponto de vista funcional, este microórgão apresenta aumento da sensibilidade à glicose, como se observa pela diminuição dos valores da EC_{50} para glicose e aumento do conteúdo total e da capacidade de secretar insulina (BONNER-WEIR et al., 1981, NOVELLI et al., 1999). Morfologicamente, ocorre hipertrofia das ilhotas pancreáticas e, de maneira ainda não totalmente esclarecida, hiperplasia das ilhotas e das células B (OGAWA et al., 1992). Assim, pode-se sugerir que durante os períodos iniciais de tratamento com dexametasona (24 e 72h) as alterações funcionais e possíveis alterações morfológicas que contribuem para o aumento da secreção de insulina pelas células B são capazes de manter, pelo menos em parte, os níveis glicêmicos dentro de limites normais. No entanto, a persistência do tratamento observada pelo período de 120h parece comprometer a sensibilidade à glicose em tecidos periféricos, visto os valores glicêmicos elevados neste período, mesmo com aumento de 6 vezes nos níveis de insulina e possíveis alterações morfológicas mencionadas acima.

Outros fatores podem contribuir para o agravamento da resistência periférica à insulina, como, por exemplo, aumento dos níveis circulantes de lipídios. Vimos que a resistência ao hormônio aumenta ao longo do tratamento como mostram os valores apresentados pelo cálculo do índice HOMA (método adequado para estudos nos quais apenas dados de jejum estão disponíveis) (GELONEZE; TAMBASCIA, 2006). Observamos aumento dos níveis plasmáticos de colesterol total após 120h da administração da dexametasona. Estes resultados estão em acordo com trabalhos prévios (TASKINEN et al. 1983; NASHEL, 1986). Foi observado aumento de colesterol total plasmático em pacientes submetidos à terapia crônica a base de glicocorticóides (NASHEL, 1986) e em pacientes portadores da Síndrome de Cushing (TASKINEN et al., 1983). Diversos autores têm proposto papel modulador dos glicocorticóides sobre os lipídios circulantes, com aumento dos níveis plasmáticos de triacilgliceróis (TG) por inibição da atividade da lipoproteína-lípase (BAGDADE et al., 1976, FRANCO-COLIN et al., 2000) e aumento dos níveis de ácidos graxos não esterificados (NEFA) pela ativação da lípase sensível a hormônio (SLAVIN; ONG; KERN, 1994). Apesar de não termos avaliado os níveis de TG e NEFA em nosso modelo, pode-se sugerir que o aumento dos níveis de insulina plasmática nos ratos DEX 24h e DEX 72h seriam suficientes para contrapor os efeitos lipolíticos do glicocorticóide. No entanto, o aumento da insulina plasmática, observada no grupo DEX 120h, parece não ser suficiente para evitar aumento dos níveis de colesterol total. Elevações dos níveis plasmáticos de TG e NEFA têm sido implicadas na patogênese da resistência periférica à insulina induzida por glicocorticóides (GUILLAUME-GENTIL; ASSIMACOPOULOS-JEANNET; JEANRENAUD, 1993; DIMITRIADIS et al., 1997; MOKUDA; SAKAMOTO, 1999).

Demonstramos aumento do conteúdo total de glicogênio e gordura hepáticos que estão em acordo com dados prévios (MOKUDA et al., 1991; SEVERINO et al., 2002). Sob a ação da dexametasona o fígado aumenta a produção de glicose hepática pela inversão da via glicolítica e por induzir formação de glicogênio hepático a partir de substratos resultantes dos processos de lipólise, proteólise e cetogênese. Assim, mesmo em jejum, os animais tratados com dexametasona apresentam maior quantidade de glicogênio por grama de tecido. A explicação para o aumento da gordura hepática não é muito precisa. Talvez, no fígado, a ação lipolítica da dexametasona não seja da mesma magnitude que nos tecidos adiposo e muscular e a síntese deste substrato possa estar acentuada nos períodos prandiais como resultado da disponibilidade elevada de ácidos graxos e aminoácidos desaminados. O aumento do conteúdo de gordura hepática tem sido associado com quadros metabólicos alterados onde a resistência à insulina é a característica predominante (MARCHESINI et al., 2001; SEVERINO et al., 2002).

Também observamos redução de massa corpórea e de ingestão alimentar após dois dias de administração da droga bem como aumento dos níveis plasmáticos de proteínas totais. A redução da massa muscular ocorre em parte como resultado da inibição da síntese protéica e aumento da proteólise (McGRATH; GOLDSPINK, 1982) juntamente com aumento do processo de gliconeogênese (SAVARY et al., 1998; SAVARY et al., 2001). Interessantemente, houve aumento da ingestão alimentar no período entre o primeiro e o segundo dia de tratamento. No entanto, a partir do segundo dia observou-se redução da ingestão com recuperação parcial no final do quinto dia de tratamento. Provavelmente, o aumento da ingestão no início do tratamento ocorre como conseqüência de alguma modulação, direta ou indireta, do glicocorticóide sobre os centros hipotalâmicos envolvidos com o controle de ingestão alimentar. A redução subseqüente do consumo alimentar pode ser explicada, parcialmente, pelo efeito anorexígeno

exercido pela insulina sobre receptores localizados no núcleo arqueado. Esta região é conhecida por estar envolvida com o controle alimentar. Injeções de insulina na região intracerebroventricular reduzem em 50% a ingestão de alimento em ratos normais confirmando tal pressuposto (TORSONI et al., 2003). Entretanto, parece ocorrer recuperação parcial da ingestão ao longo do tratamento como se observa no período de 120h. Recentemente, foi demonstrado que a administração prolongada de dexametasona em baixas concentrações (3 – 4 µg/dia) reduz peso corpóreo sem afetar a ingestão alimentar ocasionando redução de peso corpóreo preferencialmente pelo aumento do gasto calórico (PALACIOS et al., 1995; FRANCO-COLIM et al., 2000). Assim, a redução de massa corpórea observado principalmente no final do período de tratamento poderia não ser atribuído à redução de ingestão alimentar. Provavelmente a taxa metabólica específica (l O_2 . Kg^{-1} . h^{-1}) encontra-se aumentada. Estudos futuros que abordem o metabolismo energético seriam de grande valia à medida que inúmeros substratos (carboidratos, proteínas e lipídios) e hormônios (insulina, leptina, grelina, etc) envolvidos com atividade metabólica encontram-se alterados neste modelo experimental.

Tomados em conjunto, os dados obtidos no presente trabalho mostram que administração de dexametasona, 1mg/kg, induz resistência periférica à insulina e redução de peso corpóreo de maneira tempo-dependente a partir de 24h e aumento dos níveis circulantes de glicose e proteínas plasmáticos após 72h de tratamento num processo de acentuação dos efeitos pela cronificação do tratamento. O aumento dos níveis de insulina parece superar os efeitos anti-insulínicos do glicocorticóide nos dias iniciais o que não se observa após cinco dias do tratamento, mostrando a saturação da capacidade do sistema em se ajustar à nova situação imposta pelo tratamento crônico com dexametasona.

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ABSTRACT: Glucocorticoid therapies are often associated with insulin sensitivity alteration. In the present study we evaluated some metabolical parameters such as plasma glucose, insulin, protein and cholesterol levels in rats treated with dexamethasone (DEX) (1mg/kg, body weight, *ip.*) in different periods (24h, 72h and 120h). Dexamethasone-treated rats show peripherical resistance after 24h of drug administration as indicated by the fasting plasma insulin values (1.3 *vs.*6.8 ng/ml for controls [CTL] and DEX rats, respectively) and by HOMA index. Additional peripheral insulin resistance occurred until the end of treatment in DEX rats. The glycaemia remained slightly elevated until 72h period. However, marked hyperglycaemia was observed after 120h (79 *vs.*160 mg/dl for CTL and DEX rats, respectively). Significantly increase of plasma albumin and total proteins levels occurred from 72h of treatment and total cholesterol from 120h. Hepatic glycogen and hepatic fat increased in a time-dependent manner in DEX rats. Negative correlation was observed between fasting insulin and body weight values in dexamethasone-treated groups ($r > 0.95$). Therefore, dexamethasone administration, 1mg/kg, induces insulin peripheral resistance in a time-dependent manner from 24h and increase of circulating plasma glucose and proteins levels after 72h of treatment.

KEYWORDS: Carbohydrates. Glucocorticoids. Fats. Proteins. Rats. Time-dependent

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C E R T I F I C A D O

Certificamos que o Protocolo nº **1138-1**, sobre **"Avaliação dos efeitos de diferentes concentrações de dexametasona sobre parâmetros fisiológicos em ilhotas pancreáticas"**, sob a responsabilidade de **Prof. Dr. José Roberto Bosqueiro / Alex Rafacho**, 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 29 de novembro de 2006.

C E R T I F I C A T E

We certify that the protocol nº **1138-1**, entitled **"Evaluation of different concentrations of dexamethasone effects on physiological parameters in pancreatic islets"**, 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 November 29, 2006.

Campinas, 29 de novembro de 2006.

Profa. Dra. Ana Maria A. Guaraldo
Presidente

Fátima Alonso
Secretária Executiva

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