ANDRÉ OTAVIO PERES PROTZEK

INCREASED INSULIN SECRETION AND DECREASED INSULIN CLEARANCE CONTRIBUTES TO THE HYPERINSULINEMIA IN RATS AND MICE TREATED WITH GLUCOCORTICOID

AUMENTO DA SECREÇÃO E REDUÇÃO DO CLEARANCE DE INSULINA CONTRIBUEM PARA A HIPERINSULINEMIA COMPENSATÓRIA EM RATOS E CAMUNDONGOS TRATADOS COM GLICOCORTICOIDE

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ABSTRACT

Glucocorticoids (GCs) are widely used as anti-inflammatory agent, but they may induce adverse metabolic effects such as insulin resistance (IR), glucose intolerance, and occasionally, diabetes mellitus type 2. Healthy rats have been used as animal models to elucidate the islet compensatory mechanisms involved in these metabolic disturbances, and only a few studies, which have focused on the *in vivo* effects of GCs, have been conducted in mice models. Yet, whether the reduced insulin clearance also contributes to the compensatory hyperinsulinemia in GC-treated rodents is not fully understood. Here, we aimed to elucidate whether mice and rats share the pancreatic compensations that result in response to dexamethasone (DEX) treatment and also to identify the possible mechanisms that can explain its effects. Yet, we investigated whether the hyperinsulinemia induced by GC treatment in mice and rats is associated with altered hepatic insulin degrading enzyme (IDE) expression and insulin clearance. For this, male Swiss mice and Wistar rats were treated with the synthetic GC dexamethasone (1 mg/kg *b.w.*; 5 days). DEX treatment induced IR, hyperinsulinemia and dyslipidemia in both species (there was a higher magnitude in rats), but treatment had a greater effect in rats that had glucose intolerance and increased basal blood glucose compared to the control group. *Ex vivo* insulin secretion at different glucose concentrations was higher in both groups of DEX-treated rodents compared to their controls. Mice and rats showed a significant increase in β-cell mass due to increased β-cell proliferation, which was associated with upregulation of the Ir-β/AKT/mTOR and downregulation of AMPK/ACC/AS160 signaling. Insulin clearance reduced in GC-treated mice and rats, which were associated with reduced hepatic IDE expression. Thus, mice are less vulnerable than rats to the deleterious effect of GCs on glucose homeostasis. In addition, rats and mice share common islet compensations (increased β-cell function and mass) in response to GC treatment, which were associated with increased canonical and decreased non-canonical insulin signaling. Farther, the reduced insulin clearance in GC-treated rodents was, at least in part, due to reduced hepatic IDE expression, which contributed to the compensatory hyperinsulinemia. These findings corroborate the idea that pharmacological interventions that inhibit hepatic IDE may be an alternative anti-diabetic agent that helps to maintain glucose homeostasis due to hyperinsulinemia instead of hypoglycemic agent, which increase the overload in the β-cells and may lead to β-cell failure and DM2.

RESUMO

Os glicocorticoides (GC) são amplamente utilizados devido aos seus efeitos anti-inflamatórios. Porém, o tratamento com GC pode induzir efeitos deletérios sobre a homeostase glicêmica como a resistência à insulina (RI), intolerância à glicose e, dependendo do tempo e dose, pode levar a instalação do Diabetes mellitus tipo 2 (DM2). Neste sentido, ratos tem sido vastamente utilizados como modelo animal para elucidar as compensações pancreáticas envolvidas na hiperinsulinemia induzida por GC e, poucos estudos enfocando os efeitos do tratamento com GC foram realizados em camundongos. Além disso, não é completamente elucidado se a hiperinsulinemia compensatória induzida pelo tratamento com GC esta associada com alteração do clearance de insulina. Assim, nossos objetivos foram avaliar se: as compensações do pâncreas endócrino em resposta ao tratamento com GC são similares entre camundongos e ratos e, identificar possíveis mecanismos que as expliquem; e se a hiperinsulinemia compensatória induzida pelo tratamento com GC em camundongos e ratos esta associada com alterações do clearance de insulina e a expressão da proteína insuling degrading enzyme (IDE) no fígado. Para isto, camundongos Swiss e ratos Wistar machos foram tratados com o glicocorticoide sintético dexametasona (1 mg/kg *p.c.*; 5 dias consecutivos). O tratamento com GC induziu RI, hiperinsulinemia e dislipidemia em ambas as espécies, embora mais pronunciado em ratos, que também apresentaram intolerância à glicose e hiperglicemia no jejum. Ambas as espécies tratadas com GC apresentaram incremento da secreção de insulina *ex vivo* estimulada com glicose, massa e proliferação de células β, que foram associados com aumento da sinalização da via Ir-β/AKT/mTOR e redução da via AMPK/ACC/AS160 em ilhotas isoladas. O clearance de insulina reduziu em camundongos e ratos tratados com GC, o que foi associado com redução da expressão de IDE no fígado. Desta forma, nossos resultados indicam que camundongos são menos sensíveis aos efeitos deletérios do tratamento com GC sobre a homeostase glicêmica, quando comparado com ratos. Ainda, camundongos e ratos apresentam compensações pancreáticas semelhantes (incremento da função e massa de células β) em resposta ao tratamento com GC, que foi associado com aumento da sinalização da via canônica de insulina e redução da via não canônica em ilhotas isoladas. Além disso, a redução do clearance de insulina foi, ao menos em parte, devido a redução da expressão de IDE no fígado, o que contribuiu para a hiperinsulinemia compensatória em ambas as espécies tratadas com GC. Em conclusão, estes resultados corroboram a hipótese de que fármacos que inibam a expressão ou atividade da IDE no fígado podem ser uma intervenção anti-diabetogênica que auxilie na manutenção da homeostase glicêmica sem sobrecarregar as células β.

SUMÁRIO

A confusão é o começo de uma nova realidade.
(Richard Bandler)

Somos o que pensamos. Tudo o que somos surge com nossos pensamentos. Com nossos pensamentos, fazemos o nosso mundo.
(Siddharta)

AGRADECIMENTOS

Meu sonho após o mestrado era realizar um doutorado. Não qualquer doutorado. Eu anciava por realiza-lo em um laboratório onde existisse muitos pesquisadores que estudassem especificamente o diabetes, para que eu pudesse conviver e apreender neste ambiente de *know-how* específico, pois no mestrado, o departamento de fisiologia consistia de 5 professores, sendo que apenas a minha orientadora pesquisava metabolismo. E eu me realizei quando eu me vi no meio do *Laboratório 14/15*, com mais de 30 pesquisadores, sendo alguns estrangeiros, jovens pesquisadores ou *pós-docs*. A rotina era um frenesi e ficar parado dento da lab olhando as bancadas de experimento em andamento foi o meu curso intensivo diário. Sábados, domingos e feriados foram divertidos ao som de boa musica, piadas, experimentos e pausas para o café, e quando o resultado era positivo, a cerveja era nosso premio, quando dava errado, era nossa consolação.

Para esta consumação, eu devo sinceros agradecimentos ao *professor Boschero*, que aceitou me orientar e abriu as portas do lab, mesmo sem me conhecer pessoalmente. Esta foi uma grande demonstração de confiança e desprendimento. Além disso, apreender a formular uma hipótese, criar e contar uma estória seguindo uma linha de raciocínio coerente, e reescrever *papers* sentado ao lado do *Boschero* foi *O* curso de redação científica que eu mais buscava.

Eu realmente fui muito ensinado. Aquele colegas (*Zé, Fernanda, Luis, Leli, Junia, Sandra, Tiago, Flávia, Rosane, Vivian, Marta, Jean, Tarliza, Thais, Gabriel, Estela, Cláudio, Drika, Helena 1, 2 e 3, Ju, Gustavo, Everardo, Marise, Rafael, Renato, Priscila, Ana Paula, Kely e Patrícia*) foram em muitos momentos meus mestres de Western Blot, PCR, histologia, dosagem de proteína, piadas, canulação, isolamento de ilhotas (erro rude o meu, quando aceitei coletar aquelas ilhotas que sobraram na placa; foi um rato que deu 1000 ilhotas), bioquímica e fisiologia, pois eu cheguei no lab bem crú, como sushi.

Outro sonho, de criança, que realizei devido ao doutorado foi conhecer o *Exterior*. Com a bolsa de doutorado da *FAPESP*, além de permanecer 3 anos focado no doutorado, eu fui em congressos na Europa e na América do Norte. Assisti e conversei com os autores dos papers que eu lia e relia, e conheci estes como pessoas.. Foram momentos de júbilo intenso.

O contato com camundongos e ratos me ensinou muito, e é a estes pequenos e amigáveis seres que eu devo boa parte da minha gratidão (um pouco menos aos pretinhos, que sempre me mordiam). Estes doaram suas vidas para a ciência, onde nós buscamos a longevidade e a melhoria da nossa qualidade de vida.

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As conversas com meu *pai* e minh*a mãe* foram fundamentais para me manter mentalmente e emocionalmente saudável, nossa amizade se fortaleceu com a distância espacial.

Quando eu reprovei na primeira vez que participei do exame para ingresso no doutorado, não me passou pela cabeça perceber este evento como algo positivo. Hoje, eu agradeço, pois foram 6 meses de estágio intensivo antes do cronometro do doutorado ser disparado.

Os refeeres que avaliaram os papers e os membros da banca de qualificação e de defesa são cientistas que eu agradeço profundamente, pois é destes que eu recebo os *feedbacks* mais importantes para evoluir como pesquisador. Em alguns momentos receber um *feedback* a respeito de algo produzido por mim, foi como engolir um sapo de costas. Porém, durante o doutorado, compreendi que o *feedback* é apenas uma visão de uma nova perspectiva, e é saudável assimilar.

LISTA DE ABREVIATURAS E SIGLAS

ACC – Acetyl CoA Carboxylase

ADP – Adenosine Di-Phosphate

AKT – PKB, Protein Kinase B

AMP – Adenosine Mono-Phosphate

AMPK – AMP-Activated Protein Kinase

AS160 – AKT substrate with 160 kDa

ATP – Adenosine Tri-Phosphate

Ca^{2+} – Calcium

CHOL – Cholesterol

CNTF – Ciliary Neurotrophic Factor

CTL – Control

CX36 – Connexin 36

DEX – Dexamethasone

DM2 – Diabetes Mellitus Type 2

ERK – Extracellular Signal-Regulated Kinases

G6Pase – Glucose-6-Phosphatase

GAPDH – glyceraldehyde-3-Phosphate Dehydrogenase

GLUT4 – Glucose Transporter 4

GSIS – Glucose-Stimulated Insulin Secretion

IDE – Insulin Degrading Enzyme

ipGTT – Intraperitoneal Glucose Tolerance Test

ipITT – Intraperitoneal Insulin Tolerance Test

ipPTT – Intraperitoneal Pyruvate Tolerance Test

Ir-β – Insulin Receptor β Subunit

KI-67 – KI-67

KITT – Constant Rate of Glucose Decay per Minute (%/minute)

KPTT – Constant Rate of Glucose Appearance per Minute (%/minute)

MIN6 – mouse Insulinoma 6

mRNA – Messenger Ribonucleic Acid

mTOR– mammalian target of rapamycin

NEFA – Non-Sterified-Free-Fat-Acids

OGTT – Oral Glucose Tolerance Test

PCNA – Proliferating Cell Nuclear Antigen

PDX-1 – Pancreatic and Duodenal Homeobox-1

PKC – Protein Kinase C

RIA – Radioimunoassay

RT-PCR – Reverse Transcriptase-Polymerase Chain Reaction

SDS-PAGE – Sodium Sodecyl Sulfate PolyAcrylamide Gel Electrophoresis

T2DM – Diabetes Mellitus Type 2

TBS – Tris-Buffered Saline

TG – Transgenic mouse

βIrKO – Insulin β-cell insulin receptor knockout

Introdução

Introdução

O Diabetes Mellitus tipo 2 (DM2) é uma doença metabólica multifatorial, caracterizada por hiperglicemia, usualmente associada com resistência periférica à insulina (RI), disfunção da célula β e alteração no *clearance* de insulina (11, 15, 18, 23, 37).

No início do desenvolvimento do DM2, antes da instalação da hiperglicemia crônica, o aumento da demanda de insulina devido à redução da sua ação em tecidos insulinodependentes, induz o aumento da secreção pelas células β pancreáticas, induzindo hiperinsulinemia compensatória, que persiste enquanto as células β forem capazes de manter a demanda requerida (15). Os mecanismos pelos quais as células β compensam o aumento da demanda de insulina requerida e mantém a homeostase glicêmica são por meio de compensações funcionais (aumento da secreção de insulina estimulada com glicose) e estruturais (aumento da massa de células β) (28). Entretanto, durante a instalação do DM2, as células β podem se tornar inaptas a lidar com o aumento da demanda, devido a glicolipotoxicidade e entram em um processo apoptótico que culmina com a falência parcial ou total das células β (28), que é considerado o fator determinante para o DM2 (15).

Embora os glicocorticoides (GCs) sejam amplamente utilizados devido às suas propriedades imunossupressoras, o tratamento com GC pode induzir perturbações na homeostase glicêmica devido às suas propriedades catabólicas, especialmente sobre o adipócito (5, 25), induzindo RI, aumento da gliconeogênese hepática, intolerância à glucose e, dependendo da susceptibilidade genética, até ao DM2 (5, 22, 38, 39). É bem conhecido que a RI induzida pelo tratamento com GC (2, 3, 24, 32, 41) também induz uma hiperinsulinemia compensatória devido a adaptações pancreáticas semelhantes as observadas na RI induzida pela obesidade (32, 33, 35).

O estado de compensação pelas células β pancreáticas, observado durante o desenvolvimento do DM2 induzido por dieta hiperlipídica (7 semanas) (27), pode ser rapidamente mimetizado pelo tratamento com GC (5 dias) (32, 33, 35) devido à indução de RI. Neste sentido, ratos e camundongos têm sido utilizados como modelos animais para elucidar os mecanismos moleculares, funcionais e estruturais envolvidos na adaptação compensatória das ilhotas pancreáticas (7, 8, 13, 14, 16, 17, 21, 25, 26, 30-36).

Embora seja bem estabelecido que ratos tratados com GC apresentam aumento da função e da massa de células β, resultados em camundongos são conflitantes. Camundongos tratados com GC apresentaram hiperinsulinemia associada com hiperglicemia (13). Diferentemente, a secreção de insulina estimulada por glicose foi reduzida em ilhotas isoladas de camundongos tratados com GC (17, 21). Esta aparente inconsistência pode ser devido à diferenças no background genético ou à sobreposição dos efeitos direto e indireto do GC sobre a célula β.

A insulinemia plasmática é o resultado de um balanço dinâmico entre fornecimento (secreção) e remoção da insulina plasmática (clearance) (9). Neste sentido, evidências indicam que a redução do clearance de insulina também contribui para a hiperinsulinemia compensatória induzida pela obesidade em humanos (4, 19), macacos (11), cachorros (23) e ratos (40), indicando que a redução do clearance de insulina é uma compensação que contribui para a homeostase glicêmica durante o desenvolvimento do DM2 induzida pela obesidade em mamíferos. De fato, a redução do clearance de insulina precede o incremento da secreção de insulina pelas células β, em cachorros tratados com dieta hiperlipídica (23). Desta forma, é bem estabelecido que a redução do clearance de insulina é um importante fator que contribui para a hiperinsulinemia compensatória induzida pela obesidade. Porém, a associação entre redução do clearance e hiperinsulinemia não é completamente elucidada em modelo de RI induzida pelo GC.

O clearance de insulina, definido como a taxa de remoção da insulina do plasma, ocorre predominantemente no fígado (9, 10, 18), e sua degradação em hepatócitos é mediada principalmente pela insulin degrading enzyme (IDE) (9), uma proteína de 110 kDa, presente em todas as células insulinodependentes e também em células não dependentes de insulina (9). Experimentos in vitro indicam que hepatócitos tratados com GC apresentam menor interação entre IDE e insulina (12) e reduzida capacidade de degradar insulina (1). Além disso, o tratamento crônico (1 ano) com GC reduz a expressão de IDE no cérebro de macacos (20), o que esta associado com o aumento da predisposição para o desenvolvimento da doença de Alzheimer no DM2 (29). Ainda, experimentos funcionais indicam que o clearance hepático basal esta reduzido em cachorros tratados com GC (6).

Assim, o objetivo do presente trabalho foi elucidar se o tratamento com GC induz: 1) compensações pancreáticas semelhantes em ratos e camundongos e 2) alterações no clearance de insulina e na expressão hepática da IDE em ratos e camundongos.

Objetivos

Objetivos

Os objetivos específicos do presente trabalho foram elucidar se:

1) Compensações do pâncreas endócrino em resposta ao tratamento com GC são similares entre camundongos e ratos, e identificar possíveis mecanismos que as expliquem.
2) Hiperinsulinemia compensatória em modelo de RI induzida pelo tratamento com GC em camundongos e ratos esta associada a alterações no clearance de insulina e na expressão hepática de IDE.

Resultados e Discussão

Artigo 1

Parte dos resultados obtidos durante a realização deste trabalho estão apresentados a seguir sob a forma de artigo científico submetido na revista *American Journal of Physiology, Regulatory, Integrative and Comparative Physiology.*

Augmented β-cell function and mass in glucocorticoid-treated rodents are associated with increased islet Ir-β/AKT/mTOR and decreased AMPK/ACC/AS160 signaling

Running title: Pancreatic islet compensation in GC-treated rodents

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Summary

Glucocorticoids (GCs) are widely used as anti-inflammatory agents, but they may induce adverse metabolic effects, such as insulin resistance (IR), glucose intolerance, and occasionally, type 2 diabetes. Healthy rats have been used as animal models to elucidate the islet compensatory mechanisms involved in these metabolic disturbances, and only a few studies, which have focused on the *in vivo* effects of GCs, have been conducted in mice models. Here, we aimed to elucidate whether mice and rats share the pancreatic compensations that result in response to dexamethasone (DEX) treatment and also to identify the possible mechanisms that can explain its effects. Male Swiss mice and Wistar rats were treated with DEX (1 mg/kg *b.w.*; 5 days). DEX treatment induced IR, hyperinsulinemia and dyslipidemia in both species (there was a higher magnitude in rats), but treatment had a greater effect in rats that had glucose intolerance and increased basal blood glucose compared to the control group. *Ex vivo* insulin secretion at different glucose concentrations was higher in both groups of DEX-treated rodents compared to their controls. Mice and rats showed a significant increase in β-cell mass due to increased β-cell proliferation, which was associated with upregulation of the Ir-β/AKT/mTOR and downregulation of AMPK/ACC/AS160 signaling. We conclude that mice are less vulnerable than rats to the deleterious effect of GCs on glucose homeostasis. In addition, rats and mice share common islet compensations (increased β-cell function and mass) in response to GC treatment, which were associated with increased canonical and decreased non-canonical insulin signaling.

Keywords: AKT, AMPK, glucocorticoid, insulin secretion, islets.

Introduction

Glucocorticoids (GCs), such as dexamethasone (DEX), are widely prescribed in clinical practice due to their anti-inflammatory, anti-allergic and immunosuppressive properties. GCs are the standard treatment for asthma, rheumatoid arthritis, systemic lupus erythematosus, and inflammatory bowel diseases (5, 43), as well as to protect against transplanted organ rejection (1). However, supraphysiological levels of GCs (either exogenous or endogenous) induce adverse effects related to glucose homeostasis, such as decreased peripheral insulin sensitivity, glucose intolerance and dyslipidemia (7, 25, 42, 45). Depending on the genetic background, age, and time and dose of the exposure, it can also lead to type 2 diabetes *mellitus* (T2DM) (22, 25, 31, 35, 37, 45, 51).

T2DM is a multifactorial metabolic disease that is mainly characterized by hyperglycemia (41), but before overt hyperglycemia occurs, peripheral insulin resistance (IR) leads to compensatory insulin hypersecretion by pancreatic β-cells (52). This adaptive islet compensation leads to a state of hyperinsulinemia together with normoglycemia or a modest increase in glycemic values (pre-diabetic) that persist until the β-cells can handle the required demand for insulin (16). The major mechanisms by which β-cells generate hyperinsulinemia during adaptive compensation consist of functional (*e.g.,* increased insulin biosynthesis and/or secretion) and structural adaptations (*e.g.,* increased β-cell hyperplasia and hypertrophy that may result in increased β-cell mass) (16, 34, 47). Thus, when β-cells can no longer compensate, a

glucolipotoxicity process progressively develops that induces β-cell death accompanied by hypoinsulinemia, hyperglycemia and hyperlipidemia.

The state of β-cell compensation observed in T2DM that can be induced by a long-term high-fat diet (7 weeks) (33) can be rapidly mimicked by GC treatment (5 days) (37) due to the induction of peripheral IR, which is associated with increased hepatic gluconeogenesis and lipolysis in adipocytes (32).

Healthy rats and genetically obese mice have been used as laboratory models to elucidate the mechanisms (at the functional, structural and molecular levels) involved in the adaptive compensations of pancreatic islets to GC-induced IR (17, 18, 23, 37). Although it is well established that in vivo GC treatment in rats leads to increased β-cell function and mass, data in mice remain to be elucidated. DEX-treated mice exhibit hyperinsulinemia and hyperglycemia (15). In contrast, glucose-stimulated insulin secretion (GSIS) is decreased in isolated islets from DEX-treated mice (18, 23). These apparent inconsistencies may be due to the differences in the genetic background and/or the overlapping of direct and indirect effects of GC on β-cells.

Therefore, we aimed to elucidate whether the endocrine pancreas compensations in response to *in vivo* GC treatment are similar between healthy mice and rats and also to identify the possible mechanisms that can explain its effects.

Materials and methods

Reagents

Dexamethasone phosphate (Decadron) was purchased from Aché (Campinas, SP, Brazil). Human recombinant insulin (Humalin R) was obtained from Lilly (Indianapolis, IN, USA).

Trizol was purchased from Gibco-BRL (Gaithersburg, MD, USA), and Triton X-100 was purchased from Cromato Products (Diadema, SP, BR). The ^{125}I-labeled insulin used in the radioimmunoassay (RIA) was purchased from Perkin Elmer (Boston, MA, USA). SDS-PAGE and immunoblotting were performed using Bio-Rad systems (Hercules, CA, USA), and all chemicals were from Bio-Rad and Sigma Aldrich (St. Louis, MO, USA). Western blot detection of specific proteins used the following primary antibodies from Santa Cruz (Santa Cruz, CA, USA): anti-phospho-Ir-$\beta^{Tyr1162/1163}$, anti-Ir-β, anti-phospho-$AKT_{1/2/3}^{Thr308}$, anti-$AKT_{1/2/3}$, anti-phospho-ERK^{Tyr204}, anti-ERK_1, anti-PKC and anti-GAPDH. Anti-phospho-$AMPK^{Thr172}$, anti-AMPK, anti-phospho-ACC^{Ser79}, anti-ACC, anti-phospho-$AS160^{Thr642}$, anti-phospho-PKC substrates and anti-PCNA were from Cell Signaling (Temecula, CA, USA). Anti-phospho-$mTOR^{Ser2448}$ and anti-mTOR were from Abcam (Cambridge, UK), and anti-CX36 was from Invitrogen (Camarillo, CA, USA). The secondary antibodies used were anti-rabbit IgG and anti-mouse IgG from Cell Signaling. Urea anti-protease/anti-phosphatase buffer was composed of 7 M urea, 2 M thiourea, 5 mM EDTA, 1 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM sodium pyrophosphate, 2 mM PMSF, 1% Triton X-100 and 1 μg/ml aprotinin (Trasylol from Bayer Health Care Pharmaceuticals, Berkley, CA). Immunohistochemical detection of insulin was performed using an anti-insulin primary antibody (guinea pig polyclonal) (Dako, Carpinteria, CA, USA), and detection of KI-67 was performed using an anti-KI-67 antibody (Spring Bioscience, Pleasanton, CA, USA). The secondary antibodies used to detect the anti-insulin and anti-KI-67 antibodies were anti-guinea pig IgG (Invitrogen, Carlsbad, CA, USA) and HRP-conjugated anti-rabbit IgG (Nichirei Bioscience, Tokyo, JP), respectively.

Animals and experimental design

Experiments were performed on groups of male Swiss mice and male Wistar rats (80 to 100 days) obtained from the State University of Campinas Animal Breeding Center. They were maintained in appropriate animal cages and kept at 24°C on a 12:12-hours light-dark cycle. Both mice and rats had access to food and water ad libitum. The experiments with animals were approved by the Institutional State University of Campinas Committee for Ethics in Animal Experimentation under protocol number 2285-1. Mice and rats were divided into the following two groups: DEX-treated rodents (DEX) that received a daily injection of dexamethasone phosphate (*i.p.*, 1.0 mg/kg *b.w.* in 0.9% NaCl for 5 consecutive days) and control rodents (CTL) that received a daily injection of saline (*i.p.*, saline alone for 5 consecutive days) between 08:00 and 09:00. All experiments were performed 24 h after the last DEX injection (at the sixth day) to avoid the overlapping of acute and chronic effects of GCs.

Metabolic, hormonal, and biochemical measurements

Body weight was measured beginning 2 days before the start of treatment and each day thereafter until the day of euthanasia. On the day after the last DEX administration, blood was collected from the tails of a group of fasted (10-12 h) animals, and blood glucose levels were measured with a glucometer (Accu-Chek Advantage, Roche Diagnostic, Switzerland). Immediately after blood glucose determination, the animals were sacrificed (by exposure to CO_2 followed by decapitation), and the trunk blood was collected. The serum was obtained by centrifugation and was used to measure the following parameters: insulin by RIA, non-esterified-free-fatty-acids (NEFA) (Wako Chemicals; Richmont, USA), triacylglycerol (TG) and total

cholesterol (CHOL) (Roche/Hitachi; Indianapolis, IN, USA) by spectrophotometer according to the manufacturers' instructions.

Intraperitoneal insulin tolerance test (ipITT)

A separate group of fed animals received an intraperitoneal injection of insulin (1 U/kg *b.w.* in 0.9% NaCl). Blood glucose was measured at baseline (before insulin administration; 0 min) and at 5, 10, 15, 30, 45 and 60 minutes after insulin administration. Blood glucose measurements were then converted into the natural logarithm (Ln); the slope was calculated using linear regression (time × Ln[glucose]) and multiplied by 100 to obtain the constant rate of glucose decay per minute (%/minute) during the ipITT (KITT) (41).

Intraperitoneal pyruvate tolerance test (ipPTT)

A separate group of fasted (14 h) animals received an *i.p.* injection of pyruvate (1 g/kg *b.w.*). Blood glucose was measured at baseline (before pyruvate administration; 0 min) and at 5, 15, 30 and 60 min after pyruvate administration. The area-under-the-glucose-curve (A.U.C.) was obtained from the 30 min of the ipPTT after normalization of the data (37). The constant rate of glucose appearance per minute (%/minute) during the first 15 minutes of the ipPTT (KPTT) was calculated as described above.

Intraperitoneal glucose tolerance test (ipGTT)

A separate group of fasted (10 h) animals received an *i.p.* injection of 50% glucose solution (1 g/kg *b.w.*). Blood glucose was measured at baseline (before glucose administration; 0 min) and

at 15, 30, 60, 90 and 120 min after glucose administration. The A.U.C. was calculated as described above.

Islet isolation and static insulin secretion

Islets were isolated by collagenase digestion of the pancreas as described previously (40). Insulin secretion and quantification by RIA was performed using a similar method as described previously (37).

Immunohistochemistry and morphometry in the endocrine pancreas

For morphometric analysis, at least 6 pancreases from each group of mice and rats were removed, weighed and fixed for 24 hours in 4% paraformaldehyde solution, as previously described (36). For morphometry analysis, all islets present in the sections were obtained systematically by capturing images with a digital camera (Olympus DP52, Tokyo, JP) coupled to a microscope (Olympus BX51TF, Tokyo, JP). The islet, β-cell, and section areas were analyzed using the free software ImageJ (http://rsbweb.nih.gov/ij/download.html). The relative β-cell area was calculated by dividing the β-cell area per section by the total pancreas area per section, and the absolute β-cell mass was calculated by multiplying the pancreas weight by the relative β-cell area per pancreas. The relative number of islets was obtained by dividing the number of islets per section by the total area of the section (36).

β-cell proliferation

Average β-cell proliferation was obtained by counting the total islet cell nuclei stained for insulin and KI-67 using the same software cited above. β-cell proliferation was estimated by dividing the number of KI-67-positive nuclei by the total number of insulin-positive cells (36).

β-cell death

DNA was isolated from mouse or rat islets and separated into fragmented and integral subunits using the Trizol/Triton method as described previously (44). Both quantities were measured using the SYBR green method and expressed as ng/ml of DNA as previously described (44). The data are expressed as the ratio of fragmented to total DNA.

Protein extraction and immunoblotting

Protein extraction and immunoblotting were performed as previously reported (44) with minor modifications. Images were captured by the luminescent image analyzer LAS-3000 (Fujifilm, Tokyo, JP), and the specific band intensity was quantified by optical densitometry using ImageJ.

RNA isolation and quantitative RT-PCR analysis

Groups of 600 islets were homogenized in Trizol following phenol chloroform RNA extraction, as previously described (44). Relative quantities of target transcripts were calculated from duplicate samples after normalization of the data against the endogenous control, GAPDH. The primers used were as follows: PDX1 (S: aaccggaggagaataagagg and AS: gttgtcccgctactactgtt), insulin (S: ttgcagtagttctccagtt and AS: attgttccaacatggccctgt) and GAPDH (S: cctgcaccaccaactgctta and AS: gccccacggccatcacgcca).

Statistical Analysis

The results are expressed as the mean ± s.e.m. of the indicated number (n) of animals. A paired or unpaired Student's *t*-test was used for intragroup (before and after) or intergroup (CTL vs. DEX group) comparisons. All analyses were performed using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA). A *p*-value less than or equal to 0.05 was considered significant.

Results

DEX treatment reduced body and adrenal gland weights in mice and rats

It is known that 5-day DEX treatment in rats produces a dose-dependent reduction in adrenal gland mass in a marked reciprocal reduction of endogenous corticosterone concentration (37). As expected, DEX treatment induced a significant decrease in the mass of the adrenal glands in mice (30%) and rats (35%) compared with their respective controls (Table 1), which demonstrates the effectiveness of exogenous GC treatment on adrenal hypotrophy. In addition, the mice and rats showed reduced body weight (4% and 11%, respectively) (Table 1), which is a feature commonly observed in rats made insulin-resistant by DEX treatment (31, 37).

DEX treatment induced a reduction in insulin sensitivity in mice and in rats, but increased hepatic gluconeogenesis and glucose intolerance only in rats

We first confirmed the reduction in insulin sensitivity in both mice and rats. The ipITT revealed a significant reduction in insulin sensitivity in both DEX-treated groups (Figs. 1A, F, respectively) as indicated by the reduction in the KITT (Figures 1B, E), although this effect

occurred to a lesser extent in mice. We also analyzed whether GC treatment increased hepatic gluconeogenesis. DEX treatment did not alter gluconeogenesis in mice; however, DEX-treated rats showed increased glucose production in response to pyruvate administration, as indicated by the increased A.U.C. and the KPTT (Figs. 1B-D, G-I, respectively), which indicates hepatic insulin resistance. Despite a reduction in insulin sensitivity, glucose tolerance in DEX-treated mice was similar to that in their controls (see A.U.C. data; Fig. 1E). Compared to their controls, DEX-treated rats showed the well-known negative impact of GC excess on glucose tolerance (Fig. 1J), which reflects the association of increased hepatic glucose production with a possible reduction of peripheral glucose disposal.

DEX treatment induced dyslipidemia and hyperinsulinemia in both mice and rats

DEX treatment increased fasting serum cholesterol (CHOL) and triacylglycerol (TG) concentrations in both mice and rats compared to their respective controls, which indicates a negative impact of GCs on lipid metabolism in both species. In addition, DEX treatment increased the NEFA levels only in rats (Table 2), which may indicate an increased rate of lipolysis of adipose tissue. DEX-treated rodents also showed a marked increase in serum insulin levels that were 1- and 9-fold higher in mice and rats, respectively. Blood glucose was not altered in DEX-treated mice, while it was 60% higher in DEX rats compared to CTL (Table 2). Thus, the hyperinsulinemia corroborates the IR state and seems to protect against the disruption of glucose homeostasis, though GC-treated rats were glucose intolerant.

Islets from DEX-treated rats are more responsive to glucose than islets from DEX-treated

mice

Due to the increased insulinemia that was observed in both DEX-treated mice and rats, we assessed the GSIS. Compared to the control groups, isolated islets from DEX-treated rats were more responsive to all glucose concentrations used (2.8 to 22.2 mmol/L), (Figs. 2C, D), whereas islets from DEX-treated mice were more responsive to glucose up to a concentration of 11.1 mmol/L (Figs. 2A, B). These data point to species differences in the increase of β-cell function that contributes to the different degrees of hyperinsulinemia found in each species.

DEX treatment leads to increased β-cell mass in the pancreas of mice and rats

Because an increase in β-cell mass may also favor compensatory hyperinsulinemia, we investigated this parameter in DEX-treated mice and rats. Pancreatic sections stained for insulin revealed a significant increase in the number of islets per pancreatic area in both DEX-treated rats and mice compared to their respective controls (Figs. 3A, C). Additionally, DEX treatment significantly increased the absolute β-cell mass in the pancreas from both mice and rats (Fig. 3E). These data indicate a compensatory structural islet adaptation in response to DEX-induced IR in both species.

DEX treatment increases β-cell proliferation without affecting apoptosis in mouse and in rat islet cells

The β-cell mass is the result of a dynamic balance between cell death and proliferation. We found that DEX treatment significantly increased β-cell proliferation in islets from rats (420%) and mice (200%) compared with their respective controls, as indicated by the higher number of

KI-67-positive β-cell nuclei (Figs. 4A, B). In addition, DEX treatment increased the protein content of the proliferating cell nuclear antigen (PCNA) in islets to a greater extent in rats than in mice (Fig. 4C). DEX treatment did not affect apoptosis in the islets of rats or mice as judged by DNA fragmentation (Fig. 4D) and caspase-3 cleavage data (Fig. 4E).

Increased β-cell function and mass is associated with increased Ir-β/AKT and reduced AMPK/ACC pathway activities in pancreatic islets from DEX-treated mice and rats

Because insulin plays an important role in β-cell proliferation and can act directly upon the islet cells, we investigated whether insulin signaling was modulated by DEX treatment. We analyzed the canonical insulin pathway through the insulin receptor β-subunit (Ir-β) and its downstream protein, protein kinase B (AKT). In islets from DEX-treated mice, we observed an increase in p-Ir-β (Fig. 5A) without alterations in the total Ir-β protein content (Fig. 5B). In islets from DEX-treated rats, the levels of p-Ir-β and total Ir-β protein increased significantly (Figs. 5A, B). DEX-treated mice had increased islet p-AKT (Fig. 5C) without alteration of the total AKT content (Fig. 5D). In rat islets, DEX treatment resulted in higher amounts of p-AKT and total AKT (Figs. 5C, D). We also assessed whether the extracellular signal-regulated kinase (ERK) pathway, which can be activated by insulin and participates in cell proliferation and differentiation (Stork and Schmitt, 2002), is modulated by DEX-treatment. In mice islets, only p-ERK was augmented (Figs. 6E, F), while DEX-treated rat islets showed a significant increase in p-ERK and total ERK content (Figs. 6E, F). Insulin secretion can also be modulated by the non-canonical insulin pathway through the AMP-dependent protein kinase (AMPK). Islets from DEX-treated mice and rats had lower p-AMPK without alterations in the total AMPK content

(Figs. 5G, H). Acetyl-CoA carboxylase (ACC), a downstream AMPK target protein, has diminished activity when phosphorylated. In both species, DEX treatment was associated with decreased levels of phosphorylated ACC (Fig. 5I) without altering the total ACC levels in islets (Fig. 5J), which indicates increased lipid synthesis in islets. Thus, DEX treatment results in increased canonical and decreased non-canonical insulin signaling pathways in islets from mice and rats.

DEX treatment modulates proteins related to vesicle trafficking, protein synthesis, cell growth, and insulin secretion in pancreatic islets from both mice and rats

Because the AKT and AMPK pathways were modulated by DEX treatment, we analyzed the 160 kDa substrate of the serine-threonine kinase AKT (AS160), which is involved in vesicle trafficking and is also a target of AMPK. In mice and rats, DEX treatment resulted in higher p-AS160 (Fig. 6A) and lower AS160 contents (Fig. 6B) compared to the control groups. We also analyzed the mammalian target of rapamycin (mTOR), a protein that induces protein synthesis and is modulated by AKT and AMPK. Islets from DEX-treated mice and rats showed increased p-mTOR and total mTOR protein (Figs. 6C, D). Due to its importance in the insulin secretion process, we also investigated proteins related to calcium (Ca^{2+}) influx, such as protein kinase C (PKC) and connexin 36 (CX36). The levels of phosphorylated PKC-target proteins (Fig. 6E), total PKC (Fig. 6F), and CX36 were increased in islets from DEX-treated mice and rats compared to their control groups. These data indicate increased insulin secretion and β-cell mass in treated rodents.

DEX treatment is associated with increased mRNA levels of PDX1 in rat islets, but not in mice islets

We also evaluated whether DEX treatment could alter the mRNA levels of the insulin and pancreatic duodenal homeobox-1 (PDX1) genes. DEX treatment tended to increase insulin mRNA levels in pancreatic islets from both mice and rats (Fig. 6J; p= 0.056 and 0.055, respectively). In addition, DEX treatment increased PDX1 mRNA levels in rat islets, but not in mouse islets (Figure. 6J).

Discussion

As has been observed in rats, short-term GC treatment in mice reduced peripheral insulin sensitivity with compensatory hyperinsulinemia as a result of enhanced β-cell function (higher GSIS) and mass (through β-cell hyperplasia). The molecular mechanisms underlying these adaptive endocrine pancreas compensations involved an increase in the Ir/AKT/mTOR and a decrease in the AMPK/ACC/AS160 signaling pathway activities in the islets of both species.

DEX-induced alterations in insulin action as well as plasma insulin concentrations are associated with enhanced insulin secretion in response to glucose, which is frequently observed in GC-treated subjects (2, 4, 30, 50). Despite the pronounced hyperinsulinemia, which is a result of a marked compensatory GSIS and increased β-cell mass, DEX-treated rats were not able to properly counteract the peripheral insulin resistance, and both glucose intolerance and hyperglycemia developed. The results obtained in the present study with DEX-treated rats corroborated those of previous studies (17, 31, 35, 39).

For the first time, we show here that although DEX-treated mice presented a decrease in peripheral insulin sensitivity, these rodents remained normoglycemic levels and tolerance to glucose, indicating that the islet compensations (*e.g.*, increased GSIS and β-cell mass) were sufficient to prevent disruption of glucose homeostasis. Thus, at similar conditions of treatment (time and doses of DEX), mice seem to be less vulnerable than rats to the deleterious effects of GCs upon glucose homeostasis. As judged by the lower magnitude of compensatory responses in the islets, it seems that mice were less insulin-resistant than rats in response to DEX treatment. In addition, we cannot exclude the possibility that a more prolonged period and/or higher GC concentrations may produce disruptions of glucose homeostasis in mice similar to those observed in rats.

Hyperinsulinemia may be explained by several factors, including the reduction of hepatic insulin clearance (Mittelman 2000) and/or an increase of basal insulin secretion (37, 38). The data obtained regarding insulin secretion demonstrated that both DEX-treated rats and mice had higher insulin responses to glucose, including at sub-threshold glucose levels (*e.g.*, 2.8 and 5.6 mmol/L). It is well known that hydrocortisone administration acutely suppresses insulin release in mice by a mechanism that most likely involves the central activation of sympathetic nerves (24). This was not the case here, as our experiments were performed 24 h after a 5-day course of DEX, thus eliminating the possible overlapping of acute and chronic effects.

GSIS in isolated islets from *ob*/*ob* mice that were treated with DEX for 1 or 2 days (at a dose equivalent to ours) was lower compared to saline-treated *ob*/*ob* mice (18). Similarly, it was shown that 2½ days of DEX treatment (at a dose equivalent to ours) reduced insulin secretion in isolated islets from healthy as well as transgenic mice that overexpressed GC receptors

specifically in β-cells (TG-mice). In both studies, it was shown that glucose-6-phosphatase (G6Pase) activity is increased in islets from DEX-treated rats (normal and TG-mice) (18, 23) and that such alteration, by generating a futile cycle, could explain the reduced GSIS in these mice. In addition, these mice received DEX two hours prior to sacrifice (18, 23) and, as mentioned above, GCs exert an acute inhibitory effect upon *in vivo* insulin secretion (24). These two aforementioned studies have limitations that make comparison with our data difficult. First, the *ob*/*ob* mice are obese, which implies an imbalance of glucose homeostasis that may make these animals more vulnerable to DEX (18). Second, the transgenic mice model showed an exacerbated direct effect of GC treatment in β-cells because they overexpress glucocorticoid receptor (23), and it is largely known that this direct effect inhibits GSIS (13, 21, 29). Although we cannot rule out the negative impact of DEX on β-cells in DEX-treated mice, we believe that the chronic indirect effects of DEX treatment on metabolism (*e.g.*, insulin resistance) surpass the acute and/or direct effects with compensatory insulin hypersecretion.

The hyperinsulinemia in DEX-treated rodents can also be partially explained by the increased β-cell mass, which is usually observed in insulin-resistant rodents fed a high-fat (33) or high sucrose diet (11). Herein, we shown that increased β-cell mass resulted from, at least in part, increased β-cell proliferation as judged by the increased PCNA and KI-67 expression. Although increased β-cell mass and proliferation in rat pancreas has been described previously (35, 36), we are the first to demonstrate such compensatory mechanisms in mice undergoing GC treatment.

β-cell mass, proliferation and function are modulated by several stimuli through various intracellular pathways, and one of these major stimuli is insulin itself. β-cell-specific Ir-β knockout mice (βIrKO) display reduced GSIS and lower islet insulin content, and they are also

glucose intolerant, supporting the hypothesis that the loss of insulin action on β-cells leads to diabetes (20). In our study, hyperinsulinemia in both DEX-treated rodents were associated with increased p-Ir-β and p-AKT in the islets, corroborating the hypothesis that increased canonical insulin pathway (Ir-β/AKT) signaling is a common mechanisms involved in the endocrine pancreas compensation in both groups of GC-treated rodents. In addition, our results corroborate the increased islet mass and GSIS in mice overexpressing AKT1 in β-cells (49), as well as previous studies in which p-AKT was increased in DEX-treated rats (10, 39). Increased p-ERK also reinforces the hypothesis that insulin may be among the major signals to induce pancreatic islet compensations in DEX-treated rodents. In DEX-treated rats, the increased Ir-β, AKT and ERK total expression indicates an additive pancreatic compensatory mechanism compared to mice, in which the mechanism is limited to increased phosphorylation of the aforementioned proteins.

We also investigated the potential of AMPK signaling for mediation of islet adaptation in our DEX-treated mice and rats. In muscle, AMPK is an energy-sensitizing enzyme that is active at low cellular energy (increased AMP/ATP ratio) (14). In the β-cell lineage (MIN6), its inhibition by glucose is essential for the activation of the insulin secretion process (8). The decreased AMPK phosphorylation in islets from both groups of DEX-treated rodents supports the increased GSIS and may reflect the abundance of energy substrates available in the plasma. AMPK downregulation also underlies the increase in ACC activity and also suggests higher energy availability. Thus, we hypothesized that reduction in the non-canonical insulin pathway (AMPK/ACC) is a common mechanisms involved in the endocrine pancreas compensation in both groups of GC-treated rodents.

In muscle, both canonical and non-canonical insulin pathways inhibit AS160 through increased phosphorylation (19) that induces vesicle trafficking; however, the modulation of AS160 by AMPK in islets is unknown. The knockdown of AS160 in the β-cell lines increased insulin secretion at the basal glucose concentration (6). Thus, increased AS160 phosphorylation in islets from GC-treated rodents indicates that AKT, rather than the AMPK pathway, mediates AS160 inhibition, thus favoring insulin vesicle exocytosis. In addition, the reduction in the AS160 expression is another mechanism that may contribute to increased insulin secretion.

The AKT and AMPK pathways also modulate mTOR function, a kinase that integrates multiple cell signals (28, 52) and regulates β-cell function and growth (28). Our results agree with those from previous studies (27, 46) and indicate that the inhibition of AMPK and the activation of AKT synergistically activates mTOR in islets from GC-treated mice and rats, which may corroborate the increased β-cell mass and function. Another mechanism that may favor islet function is the amelioration of Ca^{2+} handling in β-cells. A Ca^{2+} influx contributes to the first (triggering) and second phase (amplifying) of insulin secretion (3). An additional increase in intracellular Ca^{2+}, under stimulatory glucose concentrations, is associated with a higher GSIS in islets from GC-treated rats (38). The higher GSIS is also associated with increased CX36 expression in islets (40), which synchronizes Ca^{2+} transit between β-cells across the islets (26). Activation of PKC, which is stimulated by Ca^{2+} and participates in the amplification of insulin secretion, is another mechanism associated with increased GSIS in rats treated with GCs (38). Here, we also observed an increase in CX36 expression and an indirect increase in PKC activity in islets from both rats and mice treated with GCs, indicating the participation of Ca^{2+} in increased β-cell function. Finally, increased expression of β-cell markers (PDX1 mRNA) only in

DEX-treated rats islets indicates that pancreatic compensations in this species also involve modifications at transcriptional levels.

In conclusion, DEX-treated rats exhibited pancreatic compensations of higher magnitude than DEX-treated mice; however, they remain glucose intolerant, indicating that rats are more vulnerable than mice to the deleterious effect of GCs on glucose homeostasis. In addition, our data demonstrate that rats and mice share common endocrine pancreas compensations (increased β-cell function and mass) in response to GC treatment, which were associated with increased Ir-β/AKT/mTOR and decreased AMPK/ACC/AS160 signaling pathways.

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Disclosures

The authors declare that there is no conflict of interest associated with this manuscript.

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Figure Captions

Figure 1. *DEX treatment induces a reduction in insulin sensitivity in mice and in rats, but increases hepatic gluconeogenesis and glucose intolerance only in rats*. **(A, F)** Blood glucose during intraperitoneal insulin tolerance test (ipITT; 1 U/Kg *b.w.*) in DEX-treated mice and rats, respectively; the inset in A and F depicts the constant rate of glucose disappearance (KITT). **(B, G)** Intraperitoneal pyruvate tolerance test (ipPTT; 1 g/Kg *b.w.*) in DEX-treated mice and rats, respectively; the inset in B and G depicts the ipPTT data normalized by minute 0**(C, H)** A.U.C and **(D, I)** the constant rate of glucose appearance (KPTT) during ipPTT in DEX-treated mice and rats, respectively. **(E, J)** intraperitoneal glucose tolerance test (ipGTT; 1 g/Kg *b.w.*) in DEX-treated mice and rats, respectively; the inset in E and J depicts the A.U.C. from ipGTT; values are mean ± S.E.M.; *n*=8-10 animals per group. *Significantly different compared to CTL. Unpaired Student's *t*-test, $p \leq 0.05$.

Figure 2. *Islets from DEX-treated rats are more responsive to glucose than islets from DEX-treated mice*. **(A, C)** Static cumulative insulin secretion in isolated islets from DEX-treated mice and rats in response to different glucose concentrations, respectively. **(B, D)** Normalized glucose-stimulated insulin secretion (GSIS) (fold increase in relation to 2.8 mmol/L glucose) in mice and in rats, respectively. Values are mean ± S.E.M.; *n*= 4-6 wells from 5 different animals; *Significantly different compared to CTL. Unpaired Student's *t*-test, $p \leq 0.05$.

Figure 3. *DEX treatment increases β-cell mass in the pancreas of mice and rats*. **(A)** Representative pancreas sections stained for insulin with Hematoxylin counterstaining. **(B)** Pancreas weight, **(C)** relative islet number per pancreas area, **(D)** relative β-cell area per pancreas

area and **(E)** absolute β-cell mass in DEX-treated mice and rats. Values are mean ± S.E.M.; *n*=5-6 animals per group (≈150 islets from mice and ≈300 islets from rats). *Significantly different compared to CTL. Unpaired Student's *t*-test, $p \leq 0.05$.

Figure 4. *DEX treatment increases β-cell proliferation without affecting apoptosis in the islets of mice and rats*. **(A)** Representative pancreas sections stained for insulin (on the top) and KI-67 (at bottom), **(B)** percentage of KI-67-positive nuclei (+) β-cell, **(C)** PCNA content, **(D)** DNA fragmentation/total DNA (ratio), and **(E)** caspase-3 cleavage/total caspase-3 ratio in islets from DEX-treated mice and rats. Values are mean ± S.E.M.; *n*=5-6 rodents per group; ≈100 islets per species (≈8500 nuclei per group). *Significantly different compared to CTL. Unpaired Student's *t*-test, $p \leq 0.05$.

Figure 5. *DEX treatment stimulates the canonical insulin pathway and inhibits the non-canonical insulin pathway in pancreatic islets from mice and rats*. **(A)** Representative immunoblotting of phosphorylated and **(B)** total Ir-β content. **(C)** Phosphorylated and **(D)** total AKT content. **(E)** Phosphorylated and **(F)** total ERK content. **(G)** Phosphorylated and **(H)** total AMPK content. **(I)** Phosphorylated and **(J)** total ACC content in islets from DEX-treated mice and rats. Values are mean ± S.E.M.; *n*=4 rodents per group.; *Significantly different compared to CTL. Unpaired Student's *t*-test, $p \leq 0.05$.

Figure 6. *DEX treatment modulates proteins related to vesicle trafficking, protein synthesis, cell growth, and insulin secretion in pancreatic islets from both mice and rats*. **(A)** Representative

immunoblotting of phosphorylated and **(B)** total AS160 content. **(C)** Phosphorylated and **(D)** total mTOR content. **(E)** Phosphorylated substrates of PKC (range from 70 to 110 kDa) and **(F)** total PKC content. **(G)** CX36 expression and **(H)** mRNA expression of INS gene and PDX1 in islets from DEX-mice and rats. Values are mean ± S.E.M.; *n*=4 rodents per group. *Significantly different compared to CTL. Unpaired Student's *t*-test, $p \leq 0.05$.

Tables

Table 1. Body and adrenal weight in DEX-treated mice and rats.

	Mice			Rats		
	Body weight (g)		Adrenal weight (mg/100g b.w.)	Body weight (g)		Adrenal weight (mg/100g b.w.)
	Before	After	After	Before	After	Before
CTL	38.7 ± 0.6	40.2 ± 0.6	18.21 ± 1.74	340.1 ± 6.5	351.2 ± 11.5	17.5 ± 1.12
DEX	39.4 ± 0.8	37.9 ± 0.7†	12.68 ± 0.72*	346.7 ± 11.2	309.6 ± 10.8†	11.38 ± 0.78*

† Significantly different using unpaired t-test vs. DEX before treatment; * Significantly different using unpaired *t*-test vs. CTL after treatment $p<0.05$; n= 6-8; Values are mean ± s.e.m.

Table 2. Metabolic variables in 12 hours-fasting DEX-treated mice and rats.

	Mice		Rats	
	CTL	DEX	CTL	DEX
Cholesterol (mg/dL)	169.7 ± 14.2	302.0 ± 18.3*	23.8 ± 1.8	30.6 ± 1.7*
Triacylglycerol (mg/dL)	106.9 ± 12.4	201.0 ± 13.8*	111.2 ± 14.2	210.1 ± 26.6*
NEFA (mmol/L)	1.2 ± 0.09	1.1 ± 0.08	0.6 ± 0.04	0.9 ± 0.09*
Glycemia (mmol/L)	5.5 ± 0.3	5.0 ± 0.4	5.5 ± 0.08	8.8 ± 0.8*
Insulinemia (pmol/L)	31.9 ± 3.09	59.9 ± 9.6*	268.6 ± 64.3	2697.4 ± 439.0*

*Significantly different using unpaired t-test versus CTL $p< 0.05$; n= 8-10;Values are mean ± s.e.m.

Figure 1

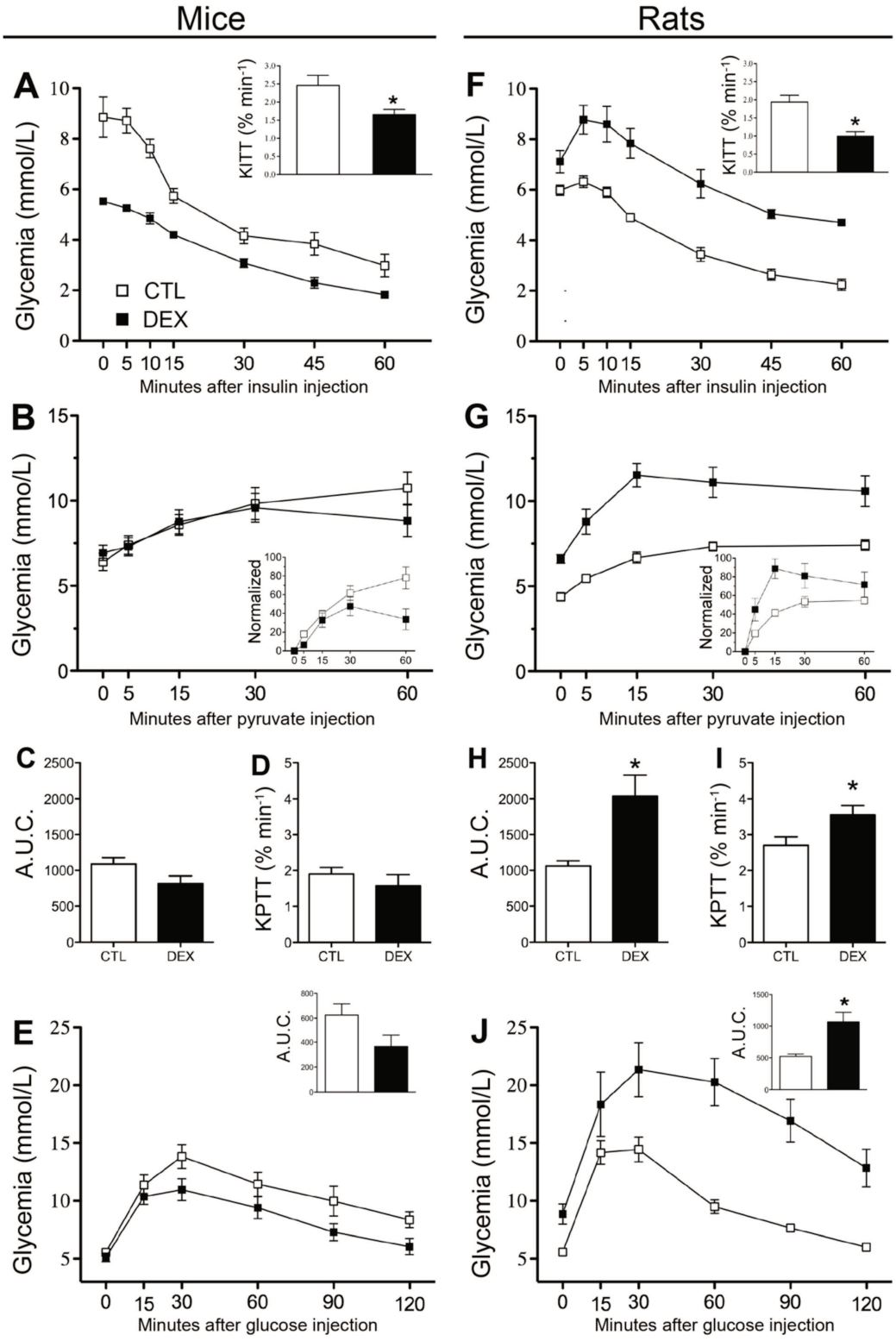

Mice
Rats
A
F
Glycemia (mmol/L)
KITT (% min-1)
CTL
DEX
Minutes after insulin injection
B
G
Glycemia (mmo/L)
Normalized
Minutes after pyruvate injection
C
H
A.U.C.
D
I
KPTT (% min-1)
E
J
Minutes after glucose injection

Figure 2

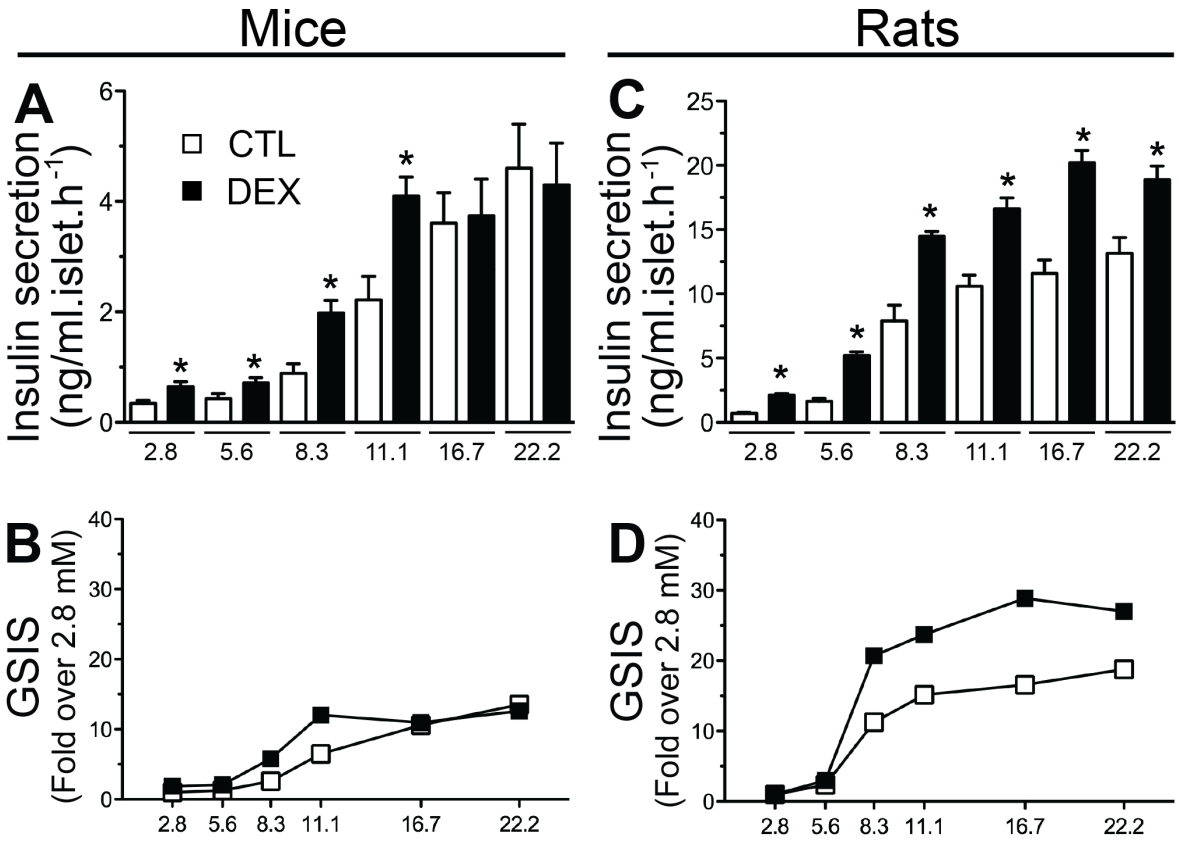

Mice
Rats
A
C
Insulin secretion (ng/ml.islet.h-1)
CTL
DEX
2.8
5.6
8.3
11.1
16.7
22.2
B
D
GSIS (Fold over 2.8 mM)

Figure 3

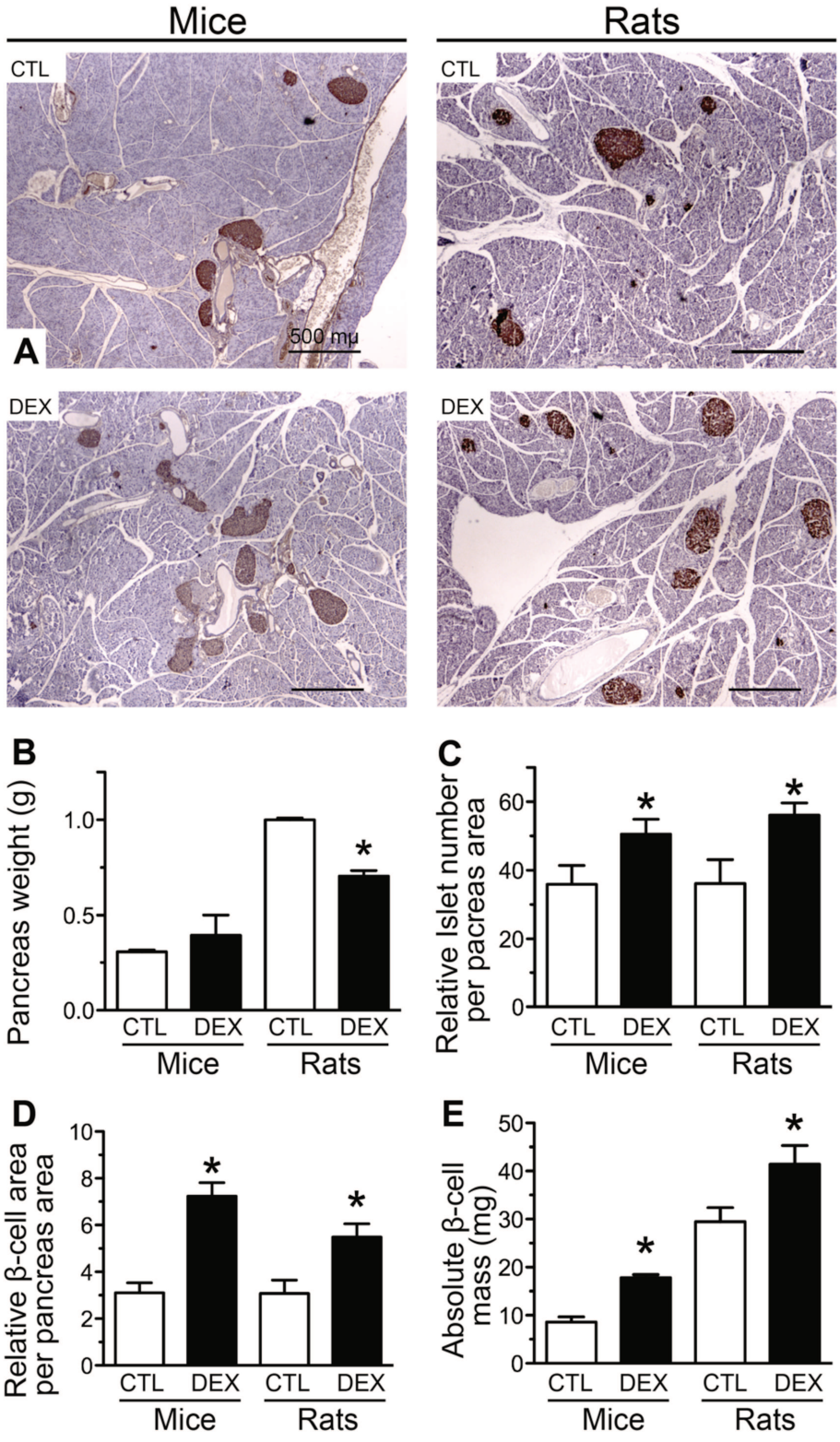

Mice
Rats
CTL
DEX
500 mμ
A
B
Pancreas weight (g)
1.0
0.5
0.0
CTL
DEX
Mice
Rats
C
Relative Islet number per pacreas area
60
40
20
0
D
Relative β-cell area per pancreas area
10
8
6
4
2
E
Absolute β-cell mass (mg)
50
40
30
20
10
*

Figure 4

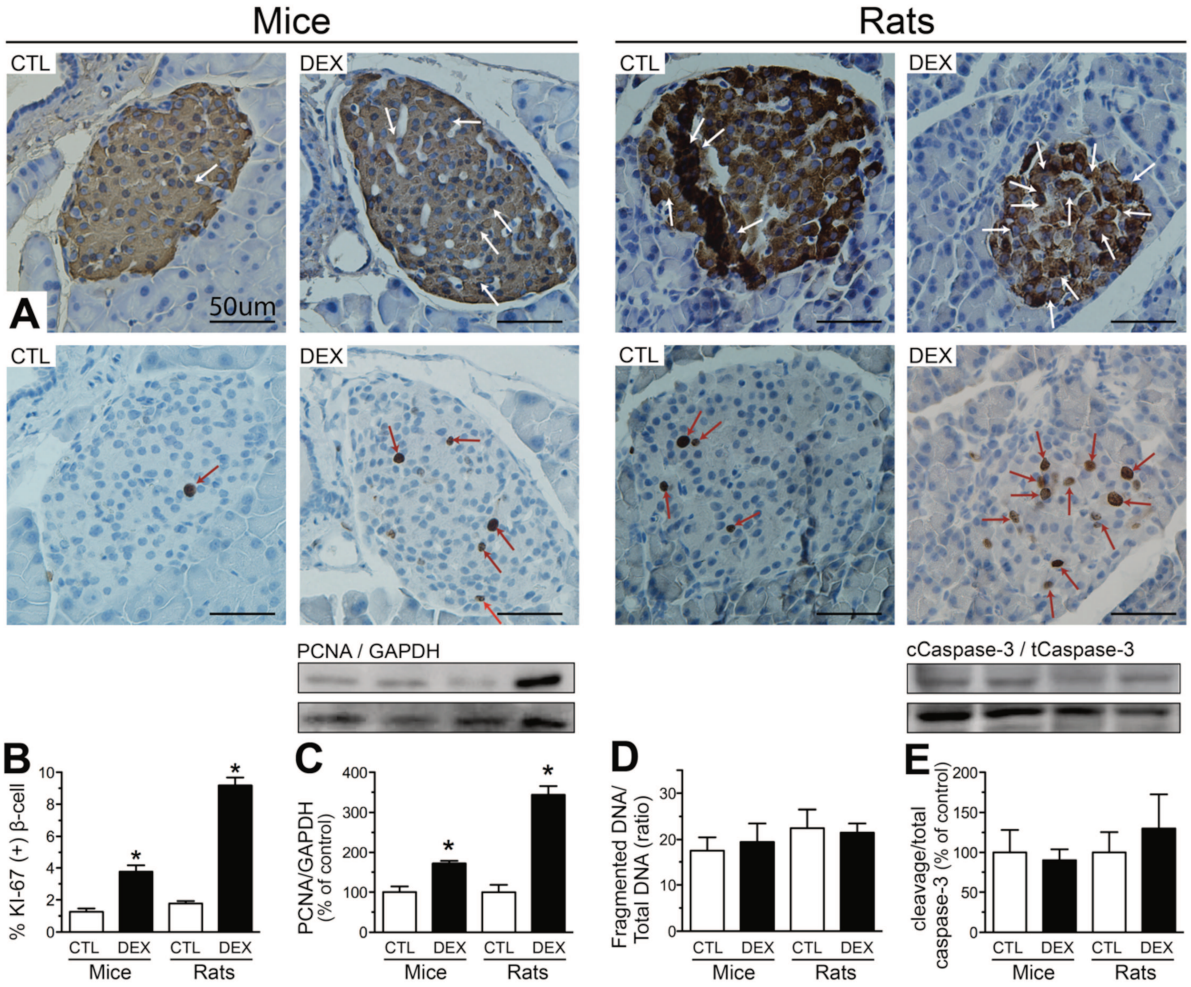

Mice
Rats
CTL
DEX
50um
A
PCNA / GAPDH
cCaspase-3 / tCaspase-3
B
% KI-67 (+) β-cell
C
PCNA/GAPDH (% of control)
D
Fragmented DNA/ Total DNA (ratio)
E
cleavage/total caspase-3 (% of control)
Mice
Rats

Figure 5

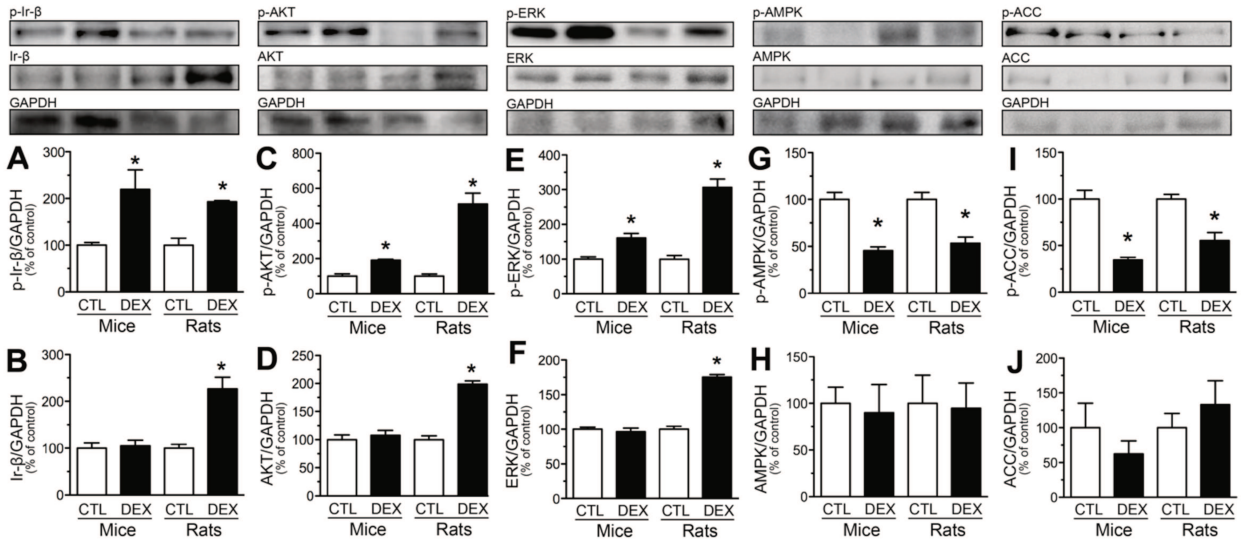
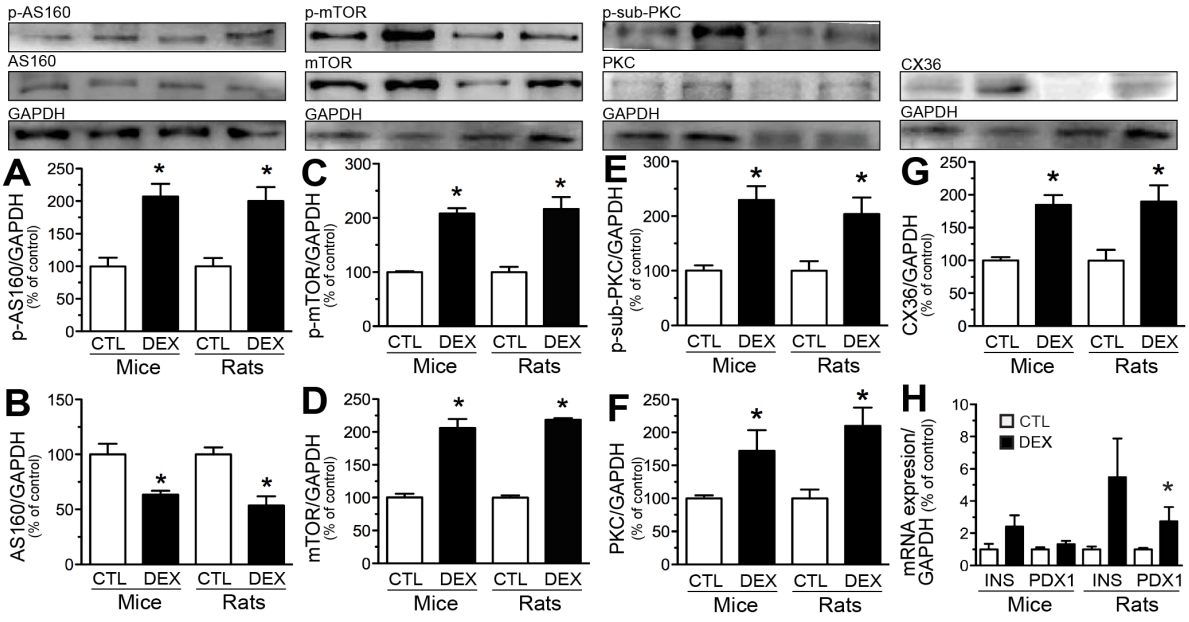

p-Ir-β
Ir-β
GAPDH
p-AKT
AKT
GAPDH
p-ERK
ERK
GAPDH
p-AMPK
AMPK
GAPDH
p-ACC
ACC
GAPDH
A
p-Ir-β/GAPDH (% of control)
B
Ir-β/GAPDH (% of control)
C
p-AKT/GAPDH (% of control)
D
AKT/GAPDH (% of control)
E
p-ERK/GAPDH (% of control)
F
ERK/GAPDH (% of control)
G
p-AMPK/GAPDH (% of control)
H
AMPK/GAPDH (% of control)
I
p-ACC/GAPDH (% of control)
J
ACC/GAPDH (% of control)
CTL DEX CTL DEX
Mice
Rats

Figure 6


p-AS160
AS160
GAPDH
p-mTOR
mTOR
GAPDH
p-sub-PKC
PKC
GAPDH
CX36
GAPDH
A
p-AS160/GAPDH (% of control)
B
AS160/GAPDH (% of control)
C
p-mTOR/GAPDH (% of control)
D
mTOR/GAPDH (% of control)
E
p-sub-PKC/GAPDH (% of control)
F
PKC/GAPDH (% of control)
G
CX36/GAPDH (% of control)
H
mRNA expresion/ GAPDH (% of control)
CTL
DEX
Mice
Rats
INS
PDX1

Artigo 2

Parte dos resultados obtidos durante a realização deste trabalho estão apresentados a seguir sob a forma de artigo científico, em submissão na revista *Journal of Endocrinology*.

Glucocorticoid treatment reduces insulin clearance in rodents by lowering hepatic IDE expression

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Short title: GC treatment reduces IDE and insulin clearance

Keywords: Dexamethasone, Glucocorticoid, insulin clearance, insulin degrading enzyme (IDE), insulin resistance

Word count: *(3492)*

Abstract

Compensatory hyperinsulinemia during the development of insulin resistance (IR), induced by treatment with glucocorticoids (GC), is due to increased insulin secretion by β-cells. However, whether reduced insulin clearance also contributes to the compensatory hyperinsulinemia is not fully understood. Thus, we aimed to investigate whether the hyperinsulinemia in insulin resistant rodents, induced by GC treatment, is associated with altered expression of insulin degrading enzyme (IDE) and insulin clearance in the liver. Accordingly, adult male Swiss mice and Wistar rats were treated with the synthetic GC dexamethasone (1 mg/kg *b.w.*; 5 days). GC treatment induced IR and hyperinsulinemia in both species, but was more impactful in rats that also displayed glucose intolerance and hyperglycemia. Insulin clearance was reduced in GC-treated mice and rats, as observed by the reduction of insulin decay rate (72% and 33%, respectively) and increased insulin area under the curve (AUC) (87% and 47%, respectively), which were associated with reduced IDE expression in the liver (62% and 37%, respectively). In conclusion, our results in insulin resistant rodents, provoked by GC treatment, support the idea that reduced insulin clearance was, at least partly, due to the reduction of the expression of hepatic IDE, which contributed to the compensatory hyperinsulinemia. These findings corroborate the idea that pharmacological interventions that inhibit IDE in the liver may be an advantageous alternative tool for maintaining glucose homeostasis, thus avoiding the use of a hypoglycemic agent that increases insulin secretion, but may cause β-cell failure and Type 2 Diabetes mellitus (T2DM).

Introduction

Type 2 Diabetes mellitus (T2DM) is characterized by hyperglycemia and is associated with obesity, peripheral insulin resistance (IR), β-cell dysfunction and altered insulin clearance (Hansen, et al. 1993; Kahn 2003; Kotronen, et al. 2008; Mittelman, et al. 2000; Rezende, et al. 2012). At the onset of T2DM, before the development of overt hyperglycemia, the IR induces an adaptive response of pancreatic β-cells that results in increased insulin secretion, leading to hyperinsulinemia. Whether this compensatory hyperinsulinemia will persist depends on the ability of β-cells to maintain this continuous requirement of insulin hypersecretion (Kahn 2003). The mechanisms by which β-cells compensate for the increased insulin demand include functional (*e.g.,* increasing insulin response to glucose) and structural adaptations (*e.g.,* expanding β-cell mass) (Poitout, et al. 2010). However, during the development of T2DM, a reduction of both β-cell function and mass may occur (Poitout et al. 2010), both of which are contributing factors for overt T2DM (Kahn 2003).

Glucocorticoids (GCs) are among several hormones with diabetogenic properties. GC-based therapies are prescribed due to their anti-inflammatory, antialergic and immunosuppressive properties. In excess, GCs promote a negative impact on glucose homeostasis due to their catabolic properties that include augmented fat lipolysis (Buren, et al. 2002; Novelli, et al. 2008), increased hepatic gluconeogenesis, IR, glucose intolerance and, depending on the individual susceptibility, T2DM (Buren et al. 2002; Longano and Fletcher 1983; Ruzzin, et al. 2005; Schacke, et al. 2002). IR induced by GC treatment is known to be initially counteracted by compensatory hyperinsulinemia (Beard, et al. 1984; Binnert, et al. 2004; Nicod, et al. 2003; Rafacho, et al. 2008a; van Raalte, et al. 2010).

A significant body of evidence indicates that hyperinsulinemia, induced during obesity, can also be generated by reduced hepatic insulin clearance in humans (Bonora, et al. 1983; Krotkiewski, et al. 1985), monkeys (Hansen et al. 1993), dogs (Mittelman et al. 2000) and rats (Stromblad and Bjorntorp 1986). A reduction in hepatic insulin clearance is a common compensatory mechanism that contributes to glucose homeostasis during the development of T2DM and is associated with obesity in mammals. In fact, decreased hepatic insulin clearance precedes the β-cell compensation in obese dogs (Mittelman et al. 2000), and this hepatic adaptive mechanism contributes to increased circulating insulin levels during obesity. However, the association between hepatic insulin clearance and hyperinsulinemia remains to be elucidated in IR induced by GC treatment.

Insulin clearance, defined as the rate of insulin removal from plasma, occurs predominantly in the liver (Duckworth, et al. 1998; Kotronen et al. 2008). Insulin degradation in hepatocytes occurs mainly by the insulin degrading enzyme (IDE), a 110 kDa zinc-metalloproteinase that is present in all insulin-sensitive and also non-sensitive cells (Duckworth et al. 1998). *In vitro* experiments indicate that hepatocytes, cultured in a medium containing GC, show a reduced interaction of insulin and IDE (Harada, et al. 1996) and a reduced insulin degrading capacity (Ali and Plas 1989). Chronic GC treatment is associated with diminished IDE expression in the brains of macaques (Kulstad, et al. 2005), which correlates with a predisposition to Alzheimer's disease in T2DM (Qiu, et al. 1998). In addition, functional experiments reveal that basal hepatic insulin clearance is reduced in GC-treated dogs (Chap, et al. 1986).

Thus, we aimed to investigate whether the hyperinsulinemia in insulin resistant rodents, induced by GC treatment, is associated with reduced hepatic insulin clearance and IDE expression in the liver.

Material and Methods

Reagents and solutions

Dexamethasone phosphate (Decadron) (Aché, Campinas, SP, Brazil), human recombinant insulin (Humalin R) (Lilly, Indianapolis, IN, USA), D-glucose (Synth, Labsynth, Diadema, SP, Brazil), Triton X-100 (Cromato Products, Diadema, SP, Brazil) and ^{125}I-labeled insulin, used in the radioimmunoassay (RIA) (Perkin Elmer, Boston, MA, USA), were used in our experiments. SDS-PAGE and immunoblotting were performed using Bio-Rad systems (Hercules, CA, USA), and all chemicals were from Bio-Rad and Sigma Aldrich (St. Louis, MO, USA). Western blot detection of IDE and GAPDH were performed with anti-IDE and anti-GAPDH (Santa Cruz Biotechnology, CA, USA) as primary antibodies, and anti-goat IgG and anti-rabbit IgG as secondary antibodies (Cell Signaling, Temecula, CA, USA). Urea anti-protease/anti-phosphatase buffer was composed of 7 mol/L urea, 2 mol/L thiourea, 5 mmol/L EDTA, 1 mmol/L sodium fluoride, 1 mmol/L sodium orthovanadate, 1 mmol/L sodium pyrophosphate, 2 mmol/L PMSF, 1% Triton X-100 and 1 μg/ml aprotinin (Trasylol) (Bayer Health Care Pharmaceuticals, Berkeley, CA, USA).

Animals and experimental design

Animal experiments were approved by the Institutional State University of Campinas Committee for Ethics in Animal Experimentation under protocol 2285-1. All of the experiments were performed in male Swiss mice and Wistar rats (80 to 100 days old) purchased from the State University of Campinas Animal Breeding Center. All of the rodents were maintained in appropriate animal cages and kept at 24°C on a 12:12 light dark cycle. Rodents had access to food and water *ad libitum*. Mice and rats were divided into a GC-treated group (GC) that received

5 consecutive daily intraperitoneal injections of dexamethasone phosphate (*i.p.*, 1.0 mg/kg *b.w.* in saline) and a control group (CTL) that received 5 consecutive daily injections of saline (1.0 ml/kg *b.w.*) between 08:00 to 09:00 h. To avoid overlapping of the acute and chronic GC effects, all experiments were initiated 24 hours after the last GC injection (on the sixth day).

Intraperitoneal insulin tolerance test (ipITT)

A group of conscious rodents were fasted for two hours and then injected with insulin (*i.p.* 1 U/kg *b.w.* in saline). Blood glucose was determined from the tail tip immediately before insulin administration (0 min) and 5, 10, 15, and 30 minutes after insulin administration. Glycemia was evaluated by a glucometer (Accu-Chek Advantage, Roche Diagnostic, Switzerland) and then converted into a natural logarithm (Ln); the slope was calculated using linear regression (time × Ln[glucose]) and multiplied by 100 to obtain the constant rate of glucose decay per minute (%/minute) during the ipITT (KITT) (Rezende et al. 2012). Insulinemia was measured by RIA, as previously described (Rezende et al. 2012).

In vivo insulin clearance

Insulin clearance was evaluated as previously described (Ahrén, et al. 2005). The constant rate for insulin disappearance (insulin decay) was calculated by converting insulin measurements into a natural logarithm (Ln); the slope was calculated using linear regression (time × Ln[insulin]) and multiplied by 100 to obtain the insulin decay constant rate per minute (%/min). The AUC of insulin during the *ip*ITT was calculated as previously described (Rezende et al. 2012).

Intraperitoneal glucose tolerance test (ipGTT) and insulin dynamics

A separate group of rodents received an *i.p.* injection of 50% glucose solution after 10 hours of fasting (1 g/kg *b.w.*). Blood samples (75-100 µl) were collected from the tail tip immediately before glucose administration (0 min) and after 15 and 60 min to determine glycemia and insulinemia as described before. The AUC of glucose and insulin during the *ip*GTT were calculated as previously described.

Tissue samples and Western Blot

One hour after the *ip*ITT, rodents were killed (by exposure to CO_2 followed by decapitation), and liver samples were extracted, snap-frozen in liquid nitrogen, and stored at -80ºC for subsequent protein extraction and immunoblotting, as previously described (Santos, et al. 2011) with brief modifications. Images were taken by the luminescent image analyzer LAS-3000 (Fujifilm, Tokyo, JP), and quantified by optical densitometry using the free software, ImageJ (http://rsbweb.nih.gov/ij/download.html).

Statistical Analysis

The results were expressed as the mean ± standard-error-of-the-mean (S.E.M.). An unpaired Student *t*-test was used for intergroup (CTL vs. GC group) comparisons. All analyses were performed using GraphPad Prism version 5.0 (San Diego, CA, USA). A p-value ≤ 0.05 was considered to be significant.

Results

GC treatment reduced insulin sensitivity in mice and rats

In vivo GC administration is known to induce a decrease in insulin sensitivity both in rats (Rafacho et al. 2009; Rafacho et al. 2008) and humans (Beard et al., 1984; van Raalte et al., 2010). As expected, GC-treated rats showed a 56% reduction in insulin sensitivity compared to CTL groups, as indicated by the ipITT (Fig. 1D,E) and the reduction in the KITT (Fig. 1F). Mice subjected to the same dexamethasone regimen also demonstrated a 36% reduction in insulin sensitivity (Fig. 1A,B), as can be observed by the KITT values (Fig. 1E).

Compensatory hyperinsulinemia to the GC-induced insulin resistance

Due to the reciprocal relationship between peripheral insulin sensitivity and pancreatic islet function (Kahn et al., 1993), insulin-resistant mice and rats demonstrated a compensatory increase in circulating insulin levels after 2 hours (137% and 150%, respectively) and 12 hours of fasting (243% and 297%, respectively) following GC administration, compared to their respective CTL groups (Table 1), which corroborates previous data (Jatwa and Kar 2009; Rafacho et al. 2008a; Rafacho, et al. 2008b). Glycemia was not altered in GC-treated mice; however, GC-treated rats exhibited hyperglycemia after both 2 hours (54%) and 12 hours of fasting (33%) compared to CTL rats, indicating a disruption of glucose homeostasis in GC-treated rats (Table 1).

Compensatory hyperinsulinemia did not prevent the development of glucose intolerance in rats

Next, we submitted the rodents to a glucose challenge (ipGTT). Similarly to previous observations (Rafacho et al., 2009; Rafacho et al., 2008a), GC-treated rats remained glucose

intolerant, as indicated by higher blood glucose levels and the increased glucose AUC during the ipGTT (Fig. 2C and inset). This altered glucose tolerance in GC-treated rats occurred even in the presence of higher insulin response to glucose, as judged by the higher insulin AUC during the ipGTT, compared to CTL rats (Fig. 2D and inset). GC-treated mice also had a higher insulin response to glucose when compared to their CTL group (Fig. 2B and inset), but remained glucose tolerant (Fig. 2A), indicating an adequate equilibrium between insulin sensitivity and islet function.

GC treatment reduced in vivo insulin clearance

By evaluating the systemic (venous) insulin levels, we indirectly determined the *in vivo* insulin clearance (decreased removal of insulin from plasma) and observed whether this parameter could be attenuated during the hyperinsulinemic state caused by GC treatment. Both mice and rats treated with GC were hyperinsulinemic before insulin injection (min 0). Five minutes post insulin administration, CTL mice and rats reached the same peak levels of insulin compared to their respective GC-treated groups. Figure 3 shows that plasma insulin clearance was significantly lower in GC-treated mice and rats 60 min after insulin injection, as judged by the increased AUC (87% and 47%, respectively) and by the reduced insulin decay rate (72% and 47%, respectively), compared to CTL groups. Thus, hyperinsulinemia in GC-treated rodents is associated with reduced insulin removal from plasma.

GC treatment reduced IDE expression in liver

GC-treated mice and rats showed a reduced IDE expression in liver (62% and 37%, respectively)

compared to their CTL groups (Fig. 4). Thus, reduced insulin clearance is associated with reduced IDE expression in the liver of GC-treated rodents.

Discussion

The present study provides evidence that the compensatory hyperinsulinemia in insulin-resistant mice and rats, induced by GC treatment, is associated with reduced hepatic insulin clearance due, at least in part, to a lower expression of IDE in the liver. These findings corroborate the idea that pharmacological interventions that reduce hepatic IDE expression or activity may be an alternative ant-diabetic tool (Leissring et al. 2010). Thus, instead of hypoglycemic agents (*e.g.* sulfonylureas), which increase the overload on β-cells and usually, after long-term therapy, lead to β-cell failure and T2DM (Takahashi, et al. 2007), an alternative approach such as inducing persistent hyperinsulimemia, by reducing IDE expression in the liver, will spare the β-cell. Although reduced hepatic insulin clearance was previously reported in GC-treated dogs (Chap et al. 1986), we are the first group to show that hyperinsulinemia, provoked by GC treatment, is associated with alterations in insulin clearance and expression of IDE in liver.

These data indicate decreased insulin sensitivity and compensatory hyperinsulinemia in GC-treated mice and rats (Fig. 1 and Table 1) and confirm previous observations in humans (Binnert et al. 20004; Beard et al. 1984; Van Raatle et al. 2010) and rats (Novelli et al. 2008; Rafacho et al. 2009; Rafacho et al. 2008; Rafacho et al. 2007). However, the effects of GC on peripheral insulin sensitivity in mice have still not been fully investigated. Here, we show that mice receiving a similar GC treatment, to that used to produce insulin-resistance in rats, also

showed a significant reduction in insulin sensitivity. However, the IR observed in mice was accompanied by a compensatory hyperinsulinemia sufficient to prevent the disruption of glucose homeostasis, including the hyperglycemia and glucose intolerance that is usually observed in GC-treated rats (Fig. 2). These data suggest that mice are less vulnerable to the deleterious effect of GC treatment on glucose homeostasis than rats.

As previously reported (Beard et al. 1984; Binnert et al. 2004; Karlsson, et al. 2001; Nicod et al. 2003; van Raalte et al. 2010), the hyperinsulinemia in GC-treated subjects is associated with increased insulin secretion, due to increased β-cell mass and function. Our ipGTT results confirm an enhancement of insulin secretion *in vivo* in response to glucose in GC-treated mice and rats, as judged by an increased acute insulin response to glucose and an overall increase in insulin AUC following GC administration (Fig. 2).

Hyperinsulinemia may also be generated by decreased insulin clearance, as observed in IR obese subjects (Hansen et al. 1993; Kotronen et al. 2008; Mittelman et al. 2000). After its secretion by pancreatic islets, insulin is collected in the portal vein, and approximately 50% is removed from plasma during this first passage through the liver (Duckworth et al. 1998). Approximately 80% of the total plasma insulin content is bound in hepatic insulin receptors (Hovorka, et al. 1993). Also, the liver is the primary site of insulin clearance (Duckworth, et al. 1988; Sato, et al. 1991), which indicates a role for the liver in hyperinsulinemia. As judged by the reduced insulin decay and increased insulin AUC (Fig. 3), after administration of a bolus of insulin, it became clear that the removal of insulin from plasma is reduced in GC-treated mice and rats, compared to their CTL groups. These results corroborate a previous report in which

fractional insulin clearance was reduced in short-term GC-treated dogs (Chap et al. 1986). This effect may reduce insulin secretion during the IR induced by GC and spare the pancreatic β-cells.

Different factors may alter insulin clearance (Duckworth et al. 1998). Glucose, lactate or hyperinsulinemia increase hepatic insulin clearance (Jaspan and Polonsky 1982; Kaden, et al. 1973; Pagano, et al. 1996; Shapiro, et al. 1987), whereas non-esterified free fatty acids (NEFA) decrease it (Hennes, et al. 1997; Svedberg, et al. 1991; Novelli et al. 2008). Perfused rat livers displayed reduced hepatic insulin clearance (40%) *in situ* when NEFA was added (Svedberg et al. 1991). In addition, obese rats also showed reduced hepatic insulin clearance, which was inversely correlated with the triglyceride (TG) content of the liver (Stromblad and Bjorntorp 1986). We found increased plasma cholesterol and TG levels in both GC-treated rodents and increased hepatic TG content only in GC-treated rats (data not shown), which may suggest that decreased insulin clearance in GC-treated rodents may be due, in part, to exposure of the liver to elevated plasma lipids and/or insulin levels.

However, indirect measurements of hepatic insulin clearance, using a mathematical model, indicated increased insulin clearance during an oral GTT in GC-treated humans (Kautzky-Willer, et al. 1996). This observation is in contrast with the data presented here. Furthermore, we do not know whether this increased hepatic insulin clearance, during oral GTT, may be altered by gut signals due to the glucose intake or modifications in the blood flow in the portal vein. In fact, the data presented by other authors were obtained after the administration of GC, which differs from our samples, which were assessed 24 hours after the last administration of GC. We believe that this approach avoids the overlapping of acute and chronic effects of GC administration.

Considering that the degradation of insulin in hepatocytes primarily occurs by IDE, we evaluated whether the decreased insulin clearance in GC-treated rodents could be associated with reduced expression of IDE in the liver. Herein, we show, for the first time, that *in vivo* GC treatment resulted in reduced IDE expression in liver, both in mice and in rats (Fig. 4), which is in accordance with the diminished insulin clearance and fasting hyperinsulinemia. Our results agree with a study in which the pharmacological inhibition of IDE led to increased insulin receptor autophosphorylation in cells (Leissring, et al. 2010), indicating that IDE regulates insulin signaling by a rapid degradation of internalized pools of insulin. Similarly, the reduction of IDE expression in the liver, induced by treatment with ciliary neurotrophic factor (CNTF) in alloxan-treated mice, resulted in lower insulin clearance, which in turn protected against their diabetogenic effects (Rezende et al. 2012). In this sense, hepatic cells (HEPG2), treated with CNTF, displayed reduced IDE expression and reduced insulin degradation (Rezende et al. 2012), supporting our findings in GC-treated rodents. In addition, reduced IDE mRNA and protein expression in the brain of GC-treated macaque (Kulstad et al. 2005) reinforces the hypothesis that GC-treatment diminishes insulin clearance.

In vitro experiments with hepatic cells (H35) indicate that GC treatment (24 hours) reduces insulin binding to IDE, without alterations in IDE expression (Harada et al. 1996), suggesting that GC-treatment may act on the removal of insulin by the liver by modulating IDE activity. Although alterations in IDE expression seem to be the primary mechanism for changes in hepatic insulin extraction, other factors such as altered IDE-insulin binding (Harada et al. 1996) or alternative IDE mRNA splicing (Farris, et al. 2005) may also be present. Currently, it is unknown whether the reduced expression of IDE in the liver was due to a direct action of GC

upon IDE or whether other factors such as increased plasma lipids and/or hyperinsulinemia were responsible.

In conclusion, our results suggest that compensatory hyperinsulinemia, in GC-treated rodents, may be explained, at least partially, by a reduced insulin clearance due to a lower expression of IDE in the liver. Thus, pharmacological interventions that reduce IDE expression or activity in that organ may be an alternative anti-diabetic approach.

Declaration of Interest

We declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contributions

A.O.P.P., L.F.R., A.R. and A.C.B. conceived and designed the experiments. A.O.P.P., L.F.R., J.M.C.J., S.M.F., A.P.C., F.M.P., J.C.S., and M.A.K., performed the experiments. A.O.P.P., L.F.R., A.R. and A.C.B. analyzed the data. A.O.P.P., L.F.R., A.R., A.C.B. and E.M.C. wrote and edited the paper.

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Figure legends

Figure 1. *GC treatment reduced insulin sensitivity in mice and rats*. **A,D)** Blood glucose (mg/dL) during intraperitoneal insulin tolerance test (ipITT; 1 U/Kg *b.w.*) in GC-treated mice and rats, respectively; **(B,E)** Blood glucose normalized as % of the initial moment (0 minutes) during the ipITT in GC-treated mice and rats, respectively; **(C,F)** the constant rate for glucose disappearance (KPTT) during ipITT in GC-treated mice and rats, respectively. Values are mean ± S.E.M.; *n*=6-9

rodents per group. *significantly different *from* CTL. Unpaired Student's *t*-test, $p \leq 0.05$.

Figure 2. *GC treatment induces hyperinsulinemia in mice and rats, but glucose intolerance only in rats*. **(A,C)** Blood glucose (**B,D**) and plasma insulin during intraperitoneal glucose tolerance test (ipGTT; 1 g/Kg *b.w.*) in DEX-treated mice and rats, respectively; the inset in A and C depicts the glucose AUC; in B, the inset depicts the insulinemia in another scale and the insulin AUC; in D, the inset depicts the insulin AUC; Values are mean ± S.E.M.; *n*=6-9 rodents per group. *significantly different *from* CTL. Unpaired Student's *t*-test, $p \leq 0.05$.

Figure 3. *GC treatment reduced in vivo insulin clearance in mice and rats.* **(A,D)** Plasma insulin before (0 min), and 5, 30 and 60 min after insulin injection in DEX-treated mice and rats, respectively; **(B,E)** insulin AUC during the ipITT and **(C,F)** insulin decay over 60 min; Values are mean ± S.E.M.; *n*=6-9 rodents per group. *significantly different *from* CTL. Unpaired Student's *t*-test, $p \leq 0.05$.

Figure 4. *GC treatment reduces hepatic IDE expression in mice and rats liver.* Representative immunoblotting of IDE expression in liver samples from mice and rats. Values are mean ± S.E.M.; *n*=4 rodents per group. *significantly different *from* CTL. Unpaired Student's *t*-test, $p \leq 0.05$.

Tables

Table 1. Metabolic variables in GC-treated mice and rats.

	Mice		Rats	
	CTL	GC	CTL	GC
2-hours Fasting				
Glycemia (mg/dL)	100.1 ± 5.8	92.4 ± 6.8	100.4 ± 1.5	154.2 ± 13.8*
Insulinemia (pmol/L)	400.0 ± 44.7	948.7 ± 187.0*	638 ± 118.9	1600.0 ± 114.0*
12-hours Fasting				
Glycemia (mg/dL)	146.8 ± 8.1	125.3 ± 8.2	101.4 ± 3.8	134.2 ± 8.7*
Insulinemia (pmol/L)	83.6 ± 8.5	285.5 ± 78.6*	284 ± 38.3	1128.0 ± 207.5*

*Significantly different using unpaired t-test versus CTL $p < 0.05$; n= 5-6;Values are mean ± s.e.m.

Figures

Figure 1

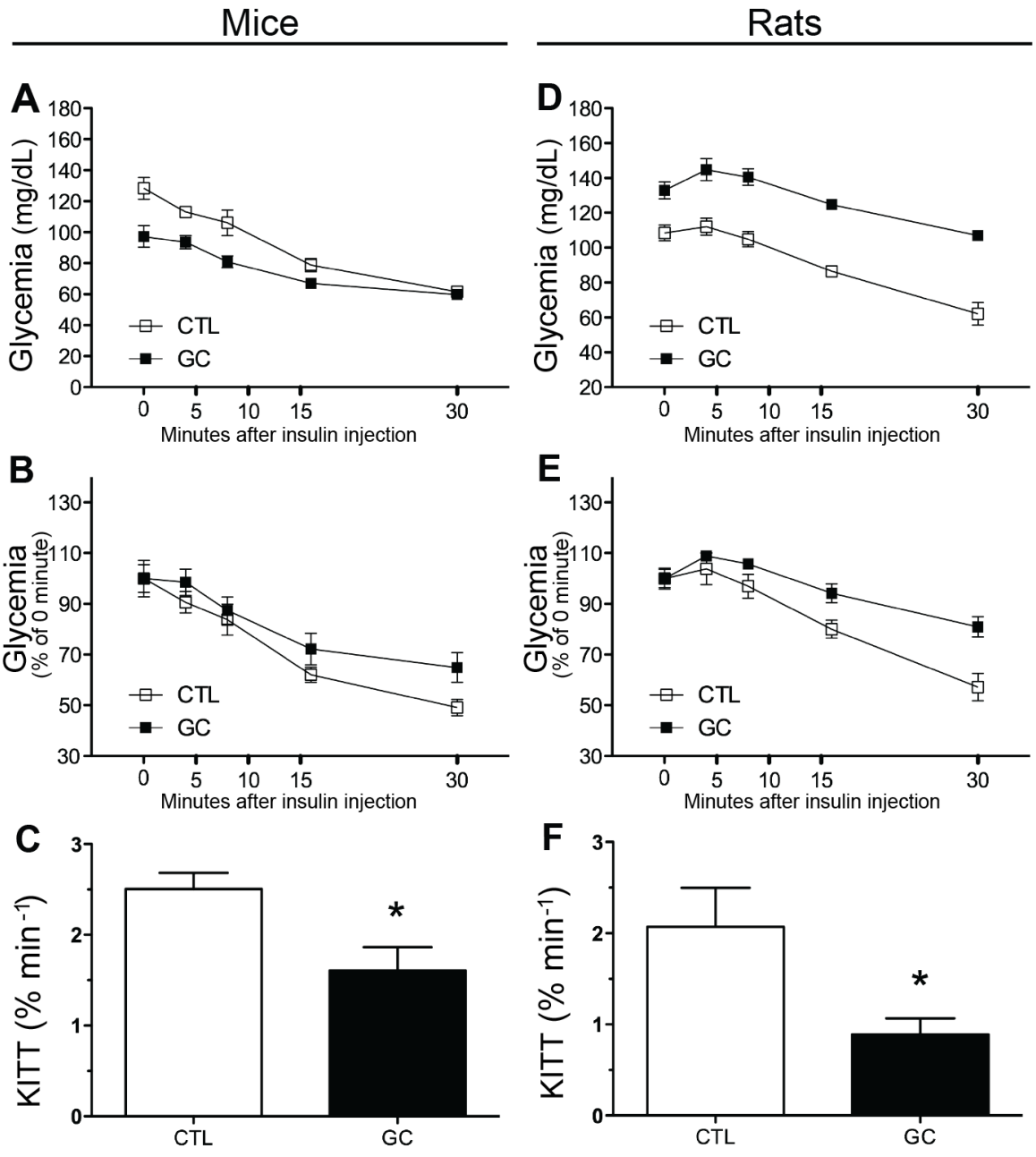


Figure 2

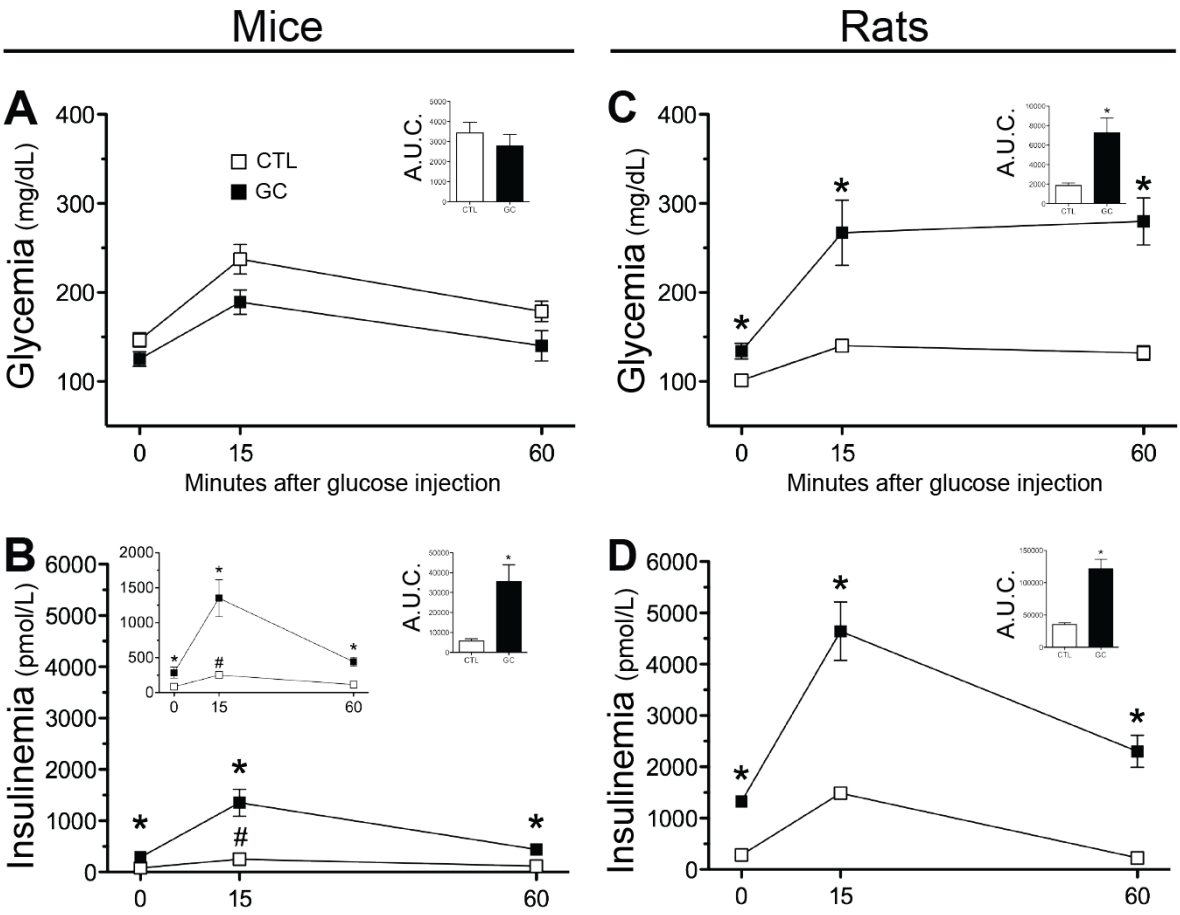

Mice
Rats
A
B
C
D
Glycemia (mg/dL)
Insulinemia (pmol/L)
CTL
GC
A.U.C.
Minutes after glucose injection
0
15
60
100
200
300
400
1000
2000
3000
4000
5000
6000
500
1500
*
#

Figure 3

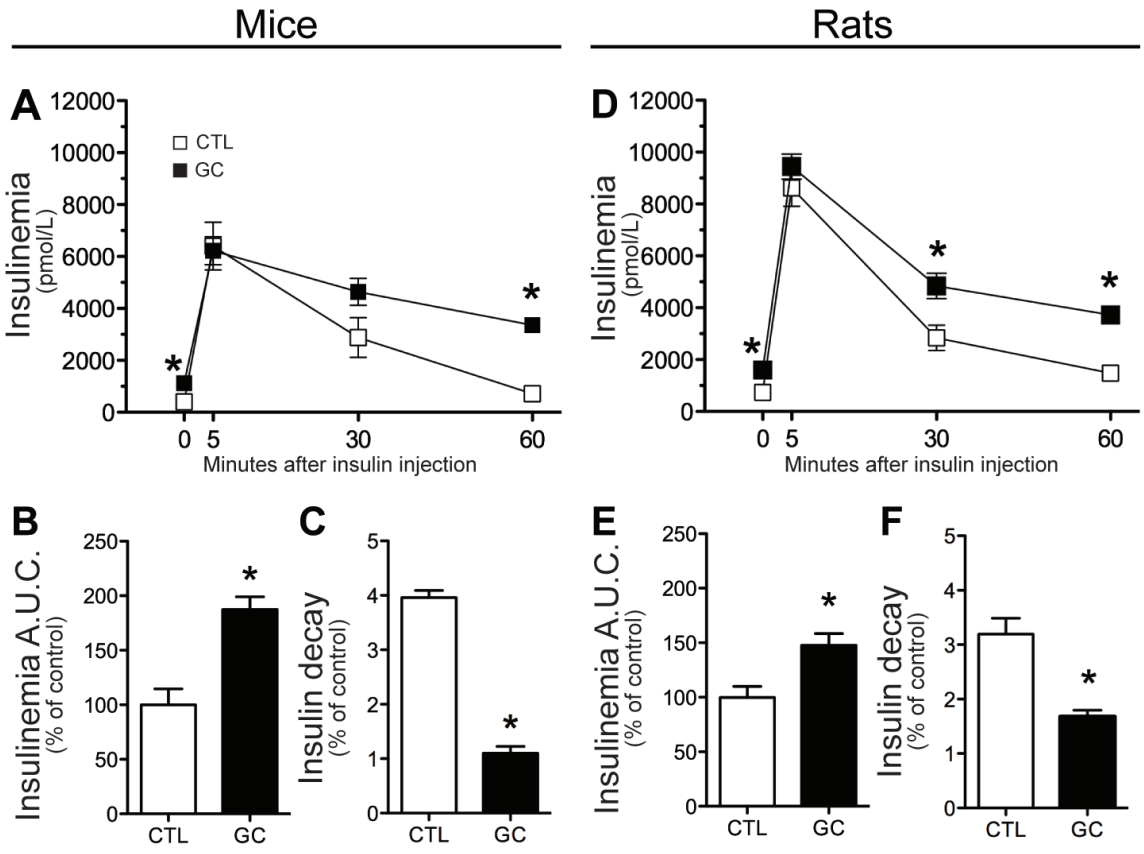
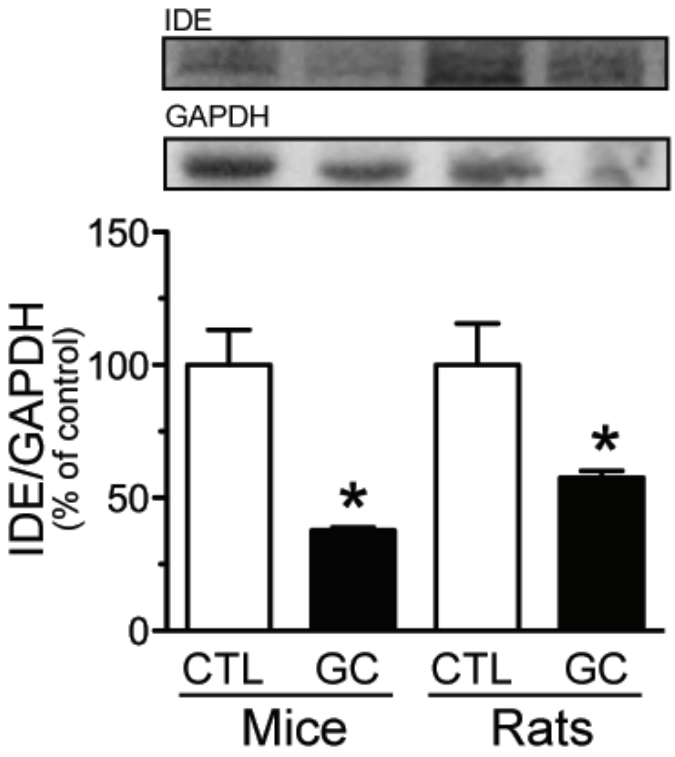

Mice
Rats
A
D
Insulinemia (pmol/L)
CTL
GC
Minutes after insulin injection
B
C
E
F
Insulinemia A.U.C. (% of control)
Insulin decay (% of control)
CTL
GC

Figure 4


IDE
GAPDH
150
100
50
0
IDE/GAPDH
(% of control)
*
*
CTL
GC
CTL
GC
Mice
Rats

Conclusões

Conclusões

Com base nos resultados obtidos no presente estudo, podemos concluir que:

1) Compensações pancreáticas em ratos tratados com GC foram de maior magnitude quando comparado com camundongos tratados com GC. Entretanto, aqueles permaneceram intolerantes à glicose, indicando que ratos são mais sensíveis aos efeitos deletérios do GC sobre a homeostase glicêmica. Além disso, nossos resultados demonstram que camundongos e ratos compartilham compensações pancreáticas semelhantes (incremento da função e da massa de células β) em resposta ao tratamento com GC, que foram associadas com incremento da sinalização da via Ir-β/AKT/mTOR e redução da sinalização da via AMPK/ACC/AS160 em ilhotas isoladas.

2) A hiperinsulinemia compensatória observada em ratos e camundongos tratados com GC pode ser explicada, pelo menos em parte, pela redução do clearance de insulina, devido à redução da expressão de IDE no fígado. Estes achados corroboram a hipótese de que intervenções farmacológias que induzem hiperinsulinemia compensatória por meio da redução da expressão ou a atividade da IDE no fígado, podem ser um agente anti-diabetogênico que auxilia na manutenção da homeostase glicêmica, ao invés de agentes hipoglicemiantes (sulfuniluréias), os quais aumentam a sobrecarga da célula β e que, após longo tempo de tratamento, geralmente induzem a falência da célula β e a instalação do DM2.

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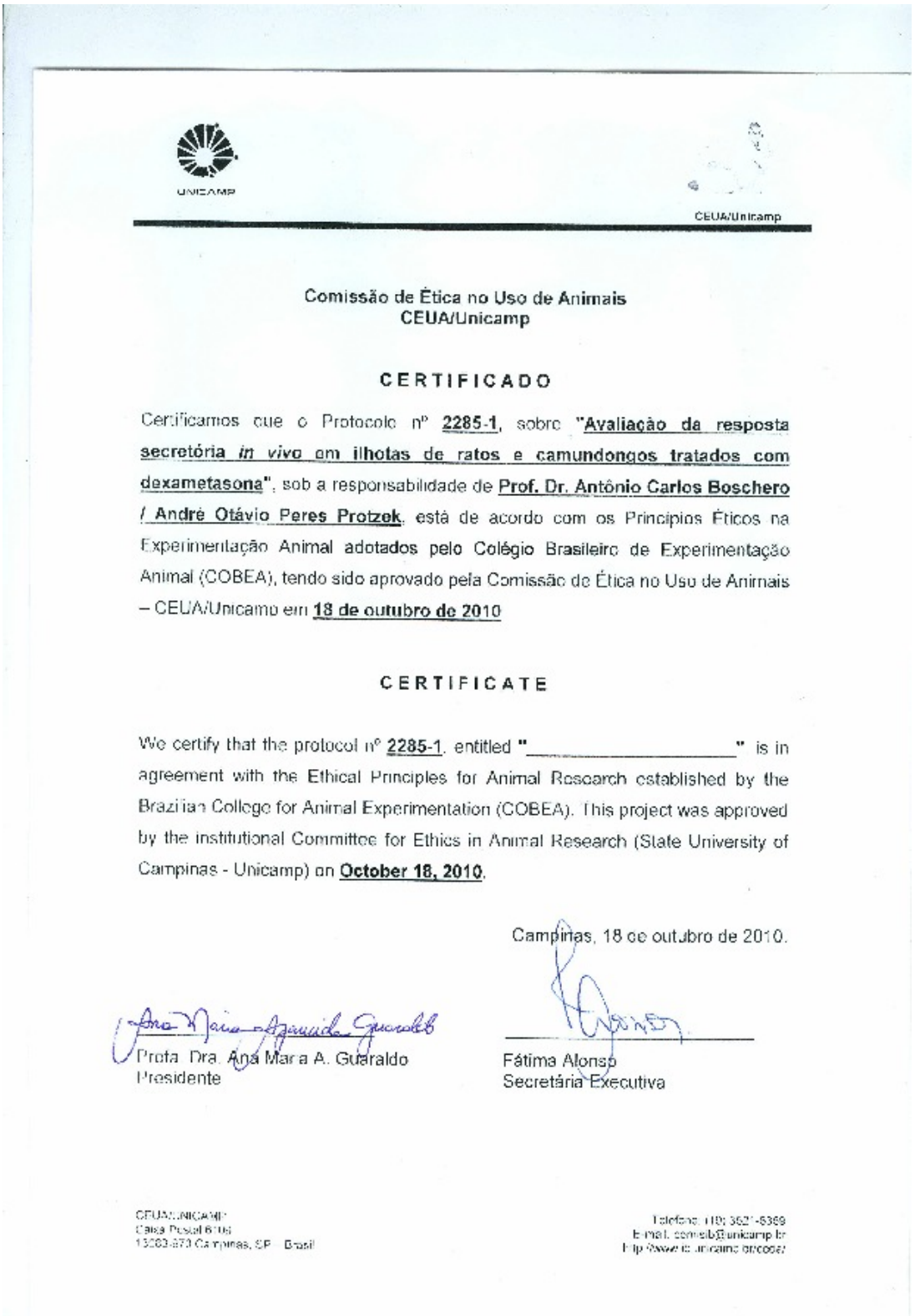
Anexos

Anexo 1



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CERTIFICADO

Certificamos que o Protocolo nº **2285-1**, sobre "**Avaliação da resposta secretória *in vivo* em ilhotas de ratos e camundongos tratados com dexametasona**", sob a responsabilidade de **Prof. Dr. Antônio Carlos Boschero / André Otávio Peres Protzek**, 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 no Uso de Animais – CEUA/Unicamp em **18 de outubro de 2010**

CERTIFICATE

We certify that the protocol nº **2285-1**, entitled "_____" is in agreement with the Ethical Principles for Animal Research established by the Brazilian College for Animal Experimentation (COBEA). This project was approved by the institutional Committee for Ethics in Animal Research (State University of Campinas - Unicamp) on **October 18, 2010**.

Campinas, 18 de outubro de 2010.

Profa. Dra. Ana Maria A. Guaraldo
Presidente

Fátima Alonso
Secretária Executiva

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Anexo 2

DECLARAÇÃO

Declaro para os devidos fins que o conteúdo de minha tese de Doutorado intitulada Aumento da secreção e redução do clearance de insulina contribuem para a hiperinsulinemia compensatória em ratos e camundongos tratados com glicocorticoide:

( ) não se enquadra no § 3º do Artigo 1º da Informação CCPG 01/08, referente a bioética e biossegurança.

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Aluno: (André Otavio Peres Protzek)

Orientador: (Antonio Carlos Boschero)

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Anexo 3

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1 message

jjones@the-aps.org <jjones@the-aps.org> Tue, Oct 8, 2013 at 11:44 AM
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Dear Mr. Protzek,

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Anexo 4

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1 message

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