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**“ENVOLVIMENTO DA VIA COLINÉRGICA NA
HIPERINSULINEMIA INDUZIDA POR GLICOCORTICÓIDE
EM RATOS”**

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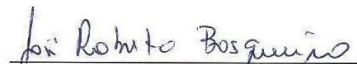
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Em adultos, a massa de célula β é mantida por um balanço entre proliferação, tamanho, neogênese e morte de célula β . A resistência à insulina (RI) perturba esta regulação e altera o crescimento e a sobrevivência destas células, promovendo um aumento compensatório da massa de células β e secreção de insulina. Neste trabalho nos investigamos a contribuição do sistema nervoso parassimpático na RI induzida por Dexametasona (DEX) e hiperinsulinemia em ratos. Ratos Wistar de 70 dias foram divididos em três grupos: Controle (CTL), vagotomizados (VAG) e sham operados (SHAM). Aos 90 dias de vida metade dos animais de cada grupo foram tratados com 1 mg/kg de DEX por 5 dias consecutivos (CTL DEX, VAG DEX e SHAM DEX). Na presença de glicose 8.3 mM mais Carbacol (Cch) 100 μ M ilhotas isoladas CTL DEX secretaram significativamente mais insulina que ilhotas CTL. Não foi observado diferença na secreção de insulina entre CTL e SHAM ou entre CTL DEX e SHAM DEX. Entretanto ilhotas de VAG DEX secretaram significativamente mais insulina. A potencialização da secreção de insulina estimulada pela glicose (SIEG) frente ao estímulo do agonista colinérgico (Cch) foi significativamente maior em ilhotas VAG CTL e VAG DEX que SHAM CTL e SHAM DEX, respectivamente. Em ilhotas CTL DEX o conteúdo das proteínas M3R e PLC β 1, mas não PKC α , foi significativamente maior comparado com seus respectivos controles. Entretanto, pPKC α aumentou significativamente em CTL DEX. Em ilhotas VAG DEX a expressão da proteína M3R aumentou significativamente comparado com VAG CTL e SHAM DEX. A vagotomia *per se* não afetou a RI, mas atenuou a insulinemia de jejum e alimentado em VAG DEX vs SHAM DEX. Em conclusão, este trabalho indica uma importante participação do sistema nervoso parassimpático através do receptor muscarínico na hiperinsulinemia induzida por DEX em ratos.

In adults, the beta-cell mass is maintained via a balance of beta-cell proliferation, size, neogenesis, and beta-cell death. The insulin-resistant state disrupts this regulation and alters beta-cell growth and survival, promoting a compensatory increase in beta-cell mass and insulin secretion. Here, we investigated the contribution of the cholinergic nervous system to dexamethasone-induced insulin resistance and hyperinsulinemia in rats. Seventy-day-old Wistar male rats were distributed in three groups, control (CTL), vagotomized (VAG), and sham operated (SHAM). On the 90th day of life, half of the rats were treated daily with 1 mg/kg of dexamethasone for 5 days (CTL DEX, VAG DEX, and SHAM DEX). In the presence of 8.3 mM glucose plus 100 μ M carbachol (Cch), isolated islets from CTL DEX secreted significantly more insulin than CTL. No differences in insulin secretion were observed between CTL and SHAM or CTL DEX and SHAM DEX. However, islets from VAG DEX secreted significantly more insulin than SHAM DEX. Cch-potentialization of secretion was further increased in islets from VAG CTL and VAG DEX than SHAM CTL and SHAM DEX, respectively. In CTL DEX islets, M3R and PLC β 1, but not PKC α , protein content was significantly higher compared with each respective control; however, pPKC α was significantly increased in the CTL DEX. In islets from VAG DEX, the expression of M3R protein increased significantly compared to VAG CTL and SHAM DEX. Vagotomy per se did not affect insulin resistance, but attenuated fasted and fed insulinemia in VAG DEX, compared with SHAM DEX rats. In conclusion, these data indicate an important participation of the cholinergic nervous system through muscaric receptors in dexamethasone-induced hyperinsulinemia in rats.

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LISTA DE ABREVIATURAS

β - beta

α_2 - **AR** - receptores adrenérgicos α_2

μg – micrograma

μl – microlitro

μM – micromolar

$[\text{Ca}^{2+}]_i$ – cálcio intracelular

$^{\circ}\text{C}$ – graus centígrados

^{125}I – insulina recombinante humana radiotiva

Ach - acetilcolina

ad libitum – à vontade

ADP – adenosina difosfato

AMPc – monofosfato de adenosina cíclico

ANOVA – análise de variância

ATP – trifosfato de adenosina

Atrop – atropina

Ca^{2+} - cálcio

CaCl₂ – cloreto de cálcio

Cch – carbacol

CO₂ – dióxido de carbono

DAG – diacilglicerol

DDT – ditiotreitol

DEPC – dietil pirocarbonato

dl – decilitro

DM2 – *Diabetes mellitus* tipo 2

falfa – animais fat/fat

IP3 – inositol 1, 4, 5 trifosfato

ipITT – teste de tolerância à insulina intraperitoneal

ipGTT - teste de tolerância à glicose intraperitoneal

K⁺ - potássio
KCl – cloreto de potássio
M3R – receptor muscarínico 3
mg/kg – miligramas/ kilogramas
MgCl₂ – cloreto de magnésio
mM – milimolar
Na₂SO₄ – sulfato de sódio
NaCl – cloreto de sódio
NaHCO₃ – bicarbonato sódio
KOH – hidróxido de potássio
ng/ml – nanogramas/mililitros
Nor – noradrenalina
ob/ob – animais obese/obese
PI – inositol fosfato
PKC – proteína cinase C
PLC – fosfolipase C
PTX – proteína G sensível à toxina pertussis
RIA – análises por radioimunoensaio
RI – Resistência à insulina
RPI – resistência periférica à insulina
S.E.M – Standard Error Mean (erro padrão da média)
SHAM – laparatomizados
SIEG – secreção de insulina estimulada pela glicose
SNA – sistema nervoso autônomo
SNS – sistema nervoso simpático
VAG – vagotomizados

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1-INTRODUÇÃO

1.1 Secreção de insulina

As ilhotas de Langerhans são microórgãos complexos, com tipos diferentes de células arraçadas estrategicamente, que possuem vascularização e inervação abundantes. Estas células sintetizam e secretam diferentes hormônios, dentre os quais a insulina possui papel relevante na regulação da homeostase glicêmica (Kahn, 1993). A secreção de insulina pelas células β pancreáticas é continuamente ajustada de acordo com as flutuações nos níveis de nutrientes circulantes, sendo a glicose o secretagogo mais importante. A secreção é ainda modulada, direta ou indiretamente, por hormônios, agentes farmacológicos e neurotransmissores, o que permite um fino ajuste nos níveis de nutrientes no sangue nas mais diversas situações fisiológicas (Boschero, 1996).

A secreção de insulina estimulada por glicose inicia-se com o reconhecimento e subsequente metabolização do nutriente pelas células β , gerando diversos sinais que levam à redução da condutância ao potássio (K^+), com consequente redução do efluxo do íon, levando à despolarização da membrana e abertura dos canais de cálcio (Ca^{2+}) sensíveis à voltagem (canais L). O influxo de Ca^{2+} e o aumento da concentração de Ca^{2+} intracelular ($[Ca^{2+}]_i$) ativa a maquinaria de exocitose, resultando na secreção de insulina. Um efeito secundário da elevação da $[Ca^{2+}]_i$ é a ativação da adenilil ciclase e fosfolipase C (PLC), que agindo sobre substratos específicos, geram AMP cíclico (AMPC), diacilglicerol (DAG) e inositol 1, 4, 5 trifosfato (IP_3). Estes amplificam o sinal da $[Ca^{2+}]_i$ por liberarem o íon de estoques celulares, e por promover a fosforilação de proteínas que sensibilizam o processo secretório do Ca^{2+} (Flatt, 1996).

1.2 Modulação da secreção de insulina pelo Sistema Nervoso Autônomo (SNA)

As ilhotas pancreáticas são ricamente innervadas por fibras adrenérgicas, colinérgicas e nervos sensoriais que, em conjunto, correspondem ao sistema nervoso autônomo (SNA), modulador as funções pancreáticas. Os neurotransmissores noradrenalina (Nor) e acetilcolina (Ach) em especial, bem como outros neuropeptídeos são os principais efetores do SNA sobre as ilhotas pancreáticas (Ahrén, 2000; Sundler; Bottcher, 1991). Têm sido demonstrado que o SNA exerce papel importante nos processos fisiológicos e fisiopatológicos associados às ilhotas pancreáticas (Ahrén, 2000).

As fibras colinérgicas têm origem pós-ganglionar, a partir de gânglios intrapancreáticos que estão sob controle de fibras pré-ganglionares que por sua vez se originam do núcleo dorsal motor do nervo vago (Brunicardi e colaboradores, 1995). As fibras vagais pós-ganglionares secretam Ach, principal neurotransmissor parassimpático, com atividade potencializadora da secreção de insulina estimulada pela glicose (SIEG) (Doyle; Egan, 2003). Este conceito está apoiado tanto por estudos *in vivo* (Bloom; Edwards, 1985) quanto por estudos *in vitro* (Verchere e colaboradores, 1991). A Ach e seu análogo carbamilcolina (Cch) agem por meio de receptores muscarínicos acoplados à proteína G (Renuka e colaboradores, 2006). Administração de Ach ou outros agonistas muscarínicos induz aumento da secreção de insulina tanto *in vivo* quanto *in vitro*, efeito inibido pela atropina (Ahrén; Karlsson, 1990).

São descritos cinco subtipos destes receptores muscarínicos, denominados de M₁ a M₅ (Jones, 1993). Os receptores muscarínicos M₁ e M₃ são os subtipos predominantes nas ilhotas pancreáticas (Zawalich e colaboradores, 1989), sendo que o receptor M₃ parece desempenhar papel preponderante no processo de potencialização da secreção de insulina (Karlsson; Ahrén, 1990). A principal característica do efeito colinérgico sobre as ilhotas ocorre por sinergia com a

glicose, resultando em potencialização da SIEG através de hidrólise de fosfoinosítídeos (PI) (Kelley e colaboradores, 1995). A hidrólise de PI de membrana resulta na formação de trifosfatidil inositol (IP_3) e diacilglicerol (DAG). IP_3 estimula liberação de Ca^{2+} a partir de estoques intracelulares no retículo endoplasmático resultando no aumento dos níveis citoplasmáticos de Ca^{2+} , independentemente da concentração extracelular de Ca^{2+} , e aumento da secreção de insulina (Niwa e colaboradores, 1998). Os íons Ca^{2+} liberados interagem com DAG e formam o complexo DAG-cálcio-fosfatidilserina que é essencial para a ativação da proteína cinase C (PKC) (Huang, 1989). A ativação da PKC promove a fosforilação de elementos fundamentais na exocitose dos grânulos de insulina potencializando a liberação do conteúdo granular para o meio extracelular (Persaud e colaboradores, 1991).

Referente à inervação adrenérgica, as fibras pré-ganglionares originam-se a partir de corpos celulares nervosos no hipotálamo e deixam a medula espinhal em nível de C8 a L3, através do nervo esplênico até os gânglios celíaco e paravertebral simpáticos, de onde emergem as fibras pós-ganglionares alcançando o pâncreas. As fibras pré-ganglionares também podem alcançar o órgão diretamente (Brunicardi e colaboradores, 1995). As fibras pós-ganglionares secretam inúmeros neurotransmissores, dentre os quais a noradrenalina, galanina e neuropeptídeo Y (Ahrén, 2000). Em geral, o sistema nervoso simpático (SNS) participa da modulação da glicemia em condições de estresse, como por exemplo, durante o exercício físico. O efeito fisiológico geral da atividade adrenérgica sobre as ilhotas pancreáticas é a inibição da SIEG, efeito mediado pela secreção de noradrenalina a partir de fibras nervosas próximas às células β com também pela elevação dos níveis plasmáticos de catecolaminas (noradrenalina e adrenalina) (Kurose e colaboradores 1990).

A ação das catecolaminas ocorre principalmente por meio de receptores α -adrenérgicos classificados em vários subtipos (Nakaki e colaboradores, 1981; Chan e colaboradores, 1997). Este fato é apoiado por estudos que demonstram que bloqueadores de receptores α -adrenérgicos podem reverter a inibição SIEG (Kurose e colaboradores, 1990) e que o uso de Clonidina (agonista específico de receptor α_2 - AR) inibe SIEG (Skoglund e colaboradores, 1988). A ativação do receptor α_2 - AR, acoplados à proteína G sensível à toxina pertussis (PTX), pela noradrenalina ou seu agonista, interfere no processo de secreção de insulina por vários mecanismos: através de hiperpolarização das células β a partir da abertura dos canais de K^+ ATP dependentes, inibindo assim o aumento da concentração de Ca^{2+} no citoplasma (Nilsson e colaboradores, 1988); pela inibição da enzima adenilato ciclase (AC), refletindo em redução da formação de AMP cíclico (AMPC) (Nakaki e colaboradores, 1981) e através da ação inibitória da maquinaria distal de exocitose (Sharp, 1996). Entretanto, noradrenalina pode também estimular a secreção de insulina através da ativação de receptores adrenérgicos β_2 e aumento de AMPC (Ahrén; Lundquist, 1981) por ativação da enzima adenilato ciclase (AC) tipo III (Samy, 1998) e da ação direta sobre as células α , provavelmente mediada por adrenoreceptores α_2 e β_2 (Chan e colaboradores, 1997, Lacey e colaboradores, 1991).

O efeito geral da estimulação adrenérgica sobre a secreção de insulina parece ocorrer em função da quantidade de α -adrenoreceptores em detrimento de β -adrenoreceptores na célula β , que podem estar alterados em diferentes condições (Ahrén, 2000). Deve ainda ser destacado que a estimulação de α_2 -adrenoreceptores pode aumentar a concentração local de noradrenalina que pode agir pré-sinápticamente e também paracrinamente (Westfall, 1984).

1.3 *Diabetes mellitus* tipo 2 (DMT2)

Segundo estimativas o DMT2 será manifestado pelo número expressivo de 366 milhões de pessoas por volta do ano 2030 (Wild e colaboradores, 2004). Componentes cruciais no desenvolvimento do diabetes mellitus como disfunções na célula β , diminuição da síntese e/ou secreção de insulina e redução da massa deste tipo celular devido ao aumento de apoptose e defeitos na regeneração têm sido descritos (Eizirik; Mandrup-Poulsen, 2000; Rhodes, 2005).

O DMT2 resulta da incapacidade das células β pancreáticas secretarem quantidades suficientes de insulina em função da demanda metabólica e assim 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 hiperglicemia permanente (Festa e colaboradores, 2006; Cnop e colaboradores, 2007). Defeitos tanto na secreção quanto na ação da insulina periféricamente 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 início da patogênese (Kahn, 2001) e é detectado por marcante redução da primeira da fase ou fase aguda da secreção de insulina estimulada por glicose (Cerasi e colaboradores, 1995; Spellman, 2007).

1.4 Resistência à Insulina (RI)

A RI geralmente está geralmente 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 responsivos à insulina como tecidos adiposo, muscular e hepático. Estudos em culturas celulares descrevem que a sinalização de

insulina, que inclui o receptor de insulina, IRS, a PI3-K, cinases dependentes de fosfatidil inositol cinase 1 (PDK1) e a proteína cinase serina/treonina Akt desempenha papel central nas ações metabólicas da insulina em muitos tipos celulares (Shepherd, 1998).

Camundongos transgênicos com deleção específica do receptor de insulina no fígado exibem RI, intolerância à glicose e ineficiência da ação da insulina sobre a supressão da produção hepática de glicose bem como sobre a regulação da expressão gênica neste tecido (Michael e colaboradores, 2000). Além do que, em estudo com camundongos em que a atividade da PI3K foi inibida especificamente no fígado como resultado da expressão desta isoforma mutada, observou-se fenótipo similar (Myiake e colaboradores, 2002).

1.4 Glicocorticóide, Resistência à Insulina e *diabetes mellitus* tipo 2

Os glicocorticóides e seus análogos sintéticos como a dexametasona fazem parte de uma classe de hormônios denominados corticosteróides, caracterizada pela habilidade de se ligar com o receptor de cortisol desencadeando efeitos similares. São amplamente utilizados no tratamento de doenças inflamatórias e autoimunes (Schaaf; Cidlowski, 2002) e produzem uma variedade de efeitos que dependem da concentração e do tempo em que são utilizados (Jeong e colaboradores, 2001). Os efeitos dos glicocorticóides sobre tecidos periféricos insulino-dependentes como o tecido muscular, adiposo e hepático são bem conhecidos (Burén e colaboradores, 2002; Ruzzin e colaboradores, 2005; Saad e colaboradores, 1995).

Os glicocorticóides são tidos como hormônios diabetogênicos por causar diminuição da captação da glicose pelos tecidos periféricos e por aumentar a produção de glicose hepática (Delaunay e colaboradores, 1997) causando a resistência periférica à insulina, provavelmente pela diminuição da fosforilação de tirosina no receptor de insulina (Ruzzin e colaboradores,

2005). A capacidade da dexametasona em induzir RI *in vivo* (Saad e colaboradores, 1993; Severino e colaboradores, 2002) e *in vitro* (Burén e colaboradores, 2002; Ruzzin e colaboradores, 2005) têm sido demonstrada e, dependendo da dose e do tempo de administração, pode ocasionar o *diabetes mellitus* tipo 2 (Pagano e colaboradores, 1983; Hoogwerf; Danese, 1999).

Ratos tratados com dexametasona (0.9 mg/kg por 2 dias) apresentam redução da captação de glicose em tecido muscular após estimulação por insulina (Weinstein e colaboradores, 1998). A redução da sensibilidade à insulina neste tecido, proveniente de ratos tratados por 12 dias consecutivos com dexametasona, também foi constatada pela diminuição da captação de glicose induzida por insulina, que está associada com redução da fosforilação da proteína Akt (Ruzzin e colaboradores, 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 e colaboradores, 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 tecido adiposo, tem sido descrita diminuição da expressão e/ou fosforilação do receptor de insulina e do IRS-1 induzida pela insulina (Saad e colaboradores, 1993). Em músculo, observou-se redução da fosforilação da Akt e da proteína glicogênio sintase cinase-3 (GSK-3) induzida pela insulina (Ruzzin e colaboradores, 2005). Estudo recente de Burén e colaboradores (2008) demonstra redução marcante tanto dos níveis de proteína quanto da fosforilação em Ser⁴⁷³ da proteína Akt em músculo e tecido adiposo de ratos submetidos a 11 dias de administração de dexametasona (1mg/kg/dia).

Durante a resistência à insulina, associada com obesidade (Holness e colaboradores, 2005) ou induzida por tratamento com glicocorticóide (Andrew; Walker, 1999), são observados normoglicemia ou moderada hiperglicemia de jejum junto com hiperinsulinemia. A RI provoca aumento dos níveis plasmáticos de insulina como consequência da hipersecreção compensatória pelas ilhotas pancreáticas de forma a manter os valores glicêmicos dentro de níveis fisiológicos (Barbera e colaboradores, 2001; Severino e colaboradores, 2002; Nicod e colaboradores, 2003). Entre as adaptações do pâncreas endócrino pode-se destacar aumento do conteúdo total de insulina em ilhotas (Bonner-Weir e colaboradores, 1981), da secreção de insulina estimulada pela glicose em ilhotas *ex vivo* (Novelli e colaboradores, 1999; Holness e colaboradores, 2005, Rafacho e colaboradores, 2007 e 2008 a,b) e aumento da massa de células β (Ogawa e colaboradores, 1992; Rafacho e colaboradores, 2008). Em contraste, no diabetes tipo 2 induzido por glicocorticóide, marcante hiperglicemia de jejum é observada juntamente com redução dos níveis de insulina sérica (Efendic e colaboradores, 1984), redução do conteúdo total de insulina, da expressão do RNAm (Toriumi; Imai, 2003) e, especialmente, diminuição da secreção de insulina induzida por glicose *in vivo* (Ohneda e colaboradores, 1993), bem como *in vitro* (Gremlich e colaboradores, 1997).

Sabe-se que a função da ilhota é reciprocamente relacionada à sensibilidade periférica à insulina, ou seja, a secreção é adaptativamente aumentada na resistência à insulina (Kahn e colaboradores, 1993). Assim, os mecanismos de resistência à insulina e/ou diabetes tipo 2 parecem estar em grande parte relacionados ao grau de funcionalidade das células β .

1.5 Hiperinsulinemia e participação das vias colinérgicas e adrenérgicas na plasticidade das Ilhotas.

A secreção de insulina é aumentada pela resistência à insulina (Ahrén; Pacini, 2005) e a falha das ilhotas em responder adequadamente com o aumento da secreção na resistência à insulina é um fator chave no desenvolvimento do diabetes tipo 2 (Larsson; Ahrén, 1996). Entretanto, os mecanismos mediadores desta compensação ainda não foram totalmente elucidados. Um mecanismo potencial que pode contribuir para a hiperinsulinemia na resistência à insulina é o aumento da potencialização da secreção de insulina estimulada por glicose (SIEG) promovida pelo do sistema nervoso autonômico (Ahrén, 2008). Sabe-se que o ramo parassimpático é um potente estimulador da secreção de insulina e que está envolvido na fase cefálica da secreção deste hormônio na alimentação (Ahrén, 2000).

O aumento da SIEG via estimulação colinérgica é observado durante condições de hiperglicemia prolongada (Balkan; Dunning, 1995; Laury e colaboradores, 1991). Dados recentes descrevem que a hipersecreção de insulina decorrente da resistência à insulina induzida por dexametasona em humanos é dependente do mecanismo autonômico (Ahrén, 2008) e que a secreção de insulina aumentada durante RI está associada ao aumento dos níveis de receptor muscarínico M3 (DelRio e colaboradores, 1997). Estudos em camundongos *ob/ob* (Ahrén; Lundquist, 1982) e ratos *fa/fa* resistentes à insulina sugeriram que a hiperinsulinemia pode ser decorrente do aumento da atividade parassimpática (Ronhner e colaboradores, 1983) e pode ser consideravelmente diminuída pelo uso da atropina (Ahren; Lundquist, 1982) e pela vagotomia (Edvell; Lindström, 1998). Mitrani e colaboradores, 2007 (*a* e *b*) demonstraram que a exposição de animais a dieta de alto teor de carboidrato (HC) durante períodos críticos do desenvolvimento resulta em hiperinsulinemia permanente com concomitante atividade alterada do SNA, incluindo

aumento da estimulação colinérgica, revertida pela vagotomia e diminuição da atividade adrenérgica. Contrariamente, ratos alimentados com dieta contendo nível de proteína semelhante à dieta dos humanos subnutridos possuem redução na secreção de insulina, assim como aumento da sensibilidade à insulina nos tecidos periféricos (Escriva e colaboradores, 1992; Swenne e colaboradores, 1987; Ferreira e colaboradores, 2003). Animais submetidos a esse tipo de dieta apresentam atividade colinérgica diminuída e adrenérgica aumentada (Ferreira e colaboradores, 2003).

Alguns trabalhos sugerem que há modificação de algum circuito neural, induzido pela RI e, através da ação colinérgica, ocorre aumento dos níveis circulantes de insulina. Uma hipótese é que a falha de tal circuito resultaria em redução da secreção de insulina e diabetes tipo 2 (Ahrén, 2000). Kohnert e colaboradores (1999), indicaram esta possibilidade em modelo experimental de Diabetes tipo 2 em hamster, onde a disfunção da ilhota foi acompanhada por redução da inervação da ilhota.

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 modulação neural sobre células β , a responsividade desta frente à agonistas e antagonistas e as alterações nas vias moleculares do sistema colinérgico em ilhotas de ratos tratados com dexametasona não têm sido descritas. Portanto, o presente trabalho buscou investigar a participação da via colinérgica sobre a modulação da secreção de insulina em ilhotas pancreáticas de ratos resistentes à insulina induzidos pela administração de dexametasona e estender os estudos do envolvimento do SNA na manutenção da hipersecreção de insulina causada pelo uso dos glicocorticóides.

Objetivo Geral

Investigar a participação da via colinérgica sobre a modulação da secreção de insulina em ilhotas pancreáticas de ratos resistentes à insulina induzidos pela administração de dexametasona 1mg/kg.

2.1 - Objetivos Específicos

- Analisar os níveis de insulina sérica de animais alimentados tratados com dexametasona
- Analisar a participação da via colinérgica sobre a secreção de insulina estimulada pela glicose utilizando-se de agonistas e antagonistas específicos em protocolos de secreção cumulativa e dinâmica de insulina com ilhotas isoladas.
- Quantificar e analisar os níveis de proteínas envolvidas na via de sinalização colinérgica, dando ênfase para o receptor muscarínico M3 e para as proteínas da vias de sinalização – fosfolipase C (PLC), proteína quinase C (PKC α) e PKC fosforilada (p PKC α).
- Verificar de forma puntual a participação da via colinérgica, em grupos submetidos à vagotomia sub-diafragmática bilateral, sobre os parâmetros acima mencionados, acrescentando ainda a avaliação da glicemia e dos níveis de insulina sérica de jejum e do grau de resistência periférica à insulina induzida pelo glicocorticóide através do teste de tolerância à insulina (ipITT) deste animais.

Involvement of the cholinergic pathway in glucocorticoid-induced hyperinsulinemia in rats

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ABSTRACT

In adults, the beta-cell mass is maintained via a balance of beta-cell proliferation, size, neogenesis, and beta-cell death. The insulin-resistant state disrupts this regulation and alters beta-cell growth and survival, promoting a compensatory increase in beta-cell mass and insulin secretion. Here, we investigated the contribution of the cholinergic nervous system to dexamethasone-induced insulin resistance and hyperinsulinemia in rats. Seventy-day-old Wistar male rats were distributed in three groups, control (CTL), vagotomized (VAG), and sham operated (SHAM). On the 90th day of life, half of the rats were treated daily with 1 mg/kg of dexamethasone for 5 days (CTL DEX, VAG DEX, and SHAM DEX). In the presence of 8.3 mM glucose plus 100 μ M carbachol (Cch), isolated islets from CTL DEX secreted significantly more insulin than CTL. No differences in insulin secretion were observed between CTL and SHAM or CTL DEX and SHAM DEX. However, islets from VAG DEX secreted significantly more insulin than SHAM DEX. Cch-potentialization of secretion was further increased in islets from VAG CTL and VAG DEX than SHAM CTL and SHAM DEX, respectively. In CTL DEX islets, M3R and PLC β 1, but not PKC α , protein content was significantly higher compared with each respective control; however, pPKC α was significantly increased in the CTL DEX. In islets from VAG DEX, the expression of M3R protein increased significantly compared to VAG CTL and SHAM DEX. Vagotomy per se did not affect insulin resistance, but attenuated fasted and fed insulinemia in VAG DEX, compared with SHAM DEX rats. In conclusion, these data indicate an important participation of the cholinergic nervous system through muscaric receptors in dexamethasone-induced hyperinsulinemia in rats.

KeyWords: Dexamethasone, insulin resistance; insulin secretion; parasympathetic nervous system.

INTRODUCTION

In humans and rodents, insulin resistance (IR) is frequently accompanied by increased plasma insulin levels in an attempt to maintain glycemia close to physiological values (7, 25, 40). IR also increases the total pancreatic insulin content (9), as well as glucose-stimulated insulin secretion (GSIS) by islets, *ex vivo* (16, 27). These alterations are crucial, since the failure of the islets to adequately respond to IR is a key factor in the development of type 2 diabetes (21). Although insulin secretion is primarily a response to circulating glucose levels (19), the amount of insulin secreted at any time is a dynamic balance between stimulatory and inhibitory signals that act to maintain glucose homeostasis (4). It was proposed that the autonomic nervous system contributes to insulin secretion and the consequent hyperinsulinemia during IR (2).

Pancreatic islets are extensively innervated by vagal cholinergic nerves that are involved in nutrient-mediated regulation of insulin secretion (35). The parasympathetic branch of the autonomic nervous system is very important for the potentiation of the GSIS (1), which is mediated by acetylcholine (ACh) (15). ACh stimulation of β -cells is known to occur primarily through activation of muscarinic type 3 receptors (M3R) (4, 22). Studies in animals with genetic obesity, such as Zucker rats and *ob/ob* mice, and models of *fa/fa* rats, have shown an association between IR and increased parasympathetic activity, accompanied by an enlargement of the pancreatic islets that may underlie the hyperinsulinemia found in these animal models (3, 36, 41). All these adaptations are attenuated by vagotomy (13, 36).

Excess glucocorticoids can elicit IR in humans and are widely used to induce pharmacological IR in animal models. The capacity of dexamethasone to induce peripheral insulin resistance *in vivo* (7, 32, 38) and *in vitro* (11, 37) has been previously demonstrated and, depending on the dose and time of treatment, can cause type 2 diabetes (17, 29). Recently, using different doses of dexamethasone, *in vivo*, it was demonstrated that 1.0 mg/kg of this glucocorticoid leads to marked peripheral insulin resistance and decreased glucose tolerance in rats (23). Islets from these rats exhibited adaptive compensations, including increases in glucose- and other secretagogues-induced insulin secretion. It was also shown that the vagus nerve establishes a circuit between the liver and the central nervous system, leading to glucocorticoid-induced glucose intolerance. In this case, afferent fibers of the vagus nerve interface with hepatic peroxisome proliferator-activated receptor alpha ($Ppar\alpha$) expression to disrupt glucose

homeostasis in response to glucocorticoid and this can be reversed by selective hepatic vagotomy (24).

In the present study, we examined whether modulation of parasympathetic nervous system is required for the compensatory islets adaptations during dexamethasone-induced insulin resistance. This was assessed by abrogation of vagus activity through a selective bilateral subdiaphragmatic vagotomy.

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 protocols and radioimmunoassay (RIA) were from Mallinckrodt Baker and Inc. (Paris, Kentucky, France), and collagenase, Hepes, albumin, activated charcoal and dextran were from Sigma (St. Louis, MO, USA). The ¹²⁵I-labeled insulin (human recombinant) for RIA was purchased from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK). SDS-PAGE and immunoblotting apparatus and chemicals were from Bio-Rad (Hercules, CA, USA) and Sigma. Antibodies for Western Blot: anti-muscarinic receptor type 3 (M3R) (rabbit polyclonal) was from Chemicon Internacional (Temecula, CA). Anti-phospholipase C β 1 (PLC β 1) (rabbit polyclonal), anti-protein kinase C α (PKC α) (mouse polyclonal), anti-phosphorylated protein kinase C (pPKC) (mouse polyclonal), and anti- β -actin were from Santa Cruz Biotechnology (Santa Cruz, CA).

Animals and treatment

Experiments were performed on male Wistar rats obtained from the State University of Campinas Animal Breeding Center; animals were kept at 24°C on a 12 h light/dark cycle with free access to food and water. The experimental protocols were approved by the institutional State University of Campinas Committee for Ethics in Animal Experimentation and followed all the recommendations for ethical usage of animals, as stated by the Colégio Brasileiro de Experimentação Animal (COBEA). The rats were randomly assigned into 6 groups: Control

(CTL) and dexamethasone (DEX) rats that were treated with daily doses ip. of saline or 1.0 mg/kg dexamethasone for 5 days from the 90th day onward. Vagotomized (VAG) and sham operated (SHAM) rats that at the 70th day of life were given subdiaphragmatic bilateral vagotomy or only had their abdominal cavities opened and closed, respectively. On the 90th day, half of these rats were treated, as described above, for CTL and DEX; these rats constituted the VAG DEX and SHAM DEX groups.

Vagotomy

Subdiaphragmatic vagotomy was performed using a modified technique previously described by Dixon *et al.* (12). Briefly, 12 h fasted rats were anesthetized with Ketamim (10 mg/kg) and Xylasin (1ml/100Kg), and a midline incision was made to expose the peritoneal cavity. The dorsal and ventral branches of the vagus nerve, which travel through the diaphragm with the esophagus, were visualized and dissected clean. Subsequently, the nerve trunks were severed and removed, followed by removal of any remaining nerve fascicles on the serosal surface of the esophagus. The abdominal incision was sutured closed, and rats were allowed to recover. In the sham operations, the experimental surgery was replicated with all branches of the vagus left intact and touched only with the tip of a moistened cotton swab. At the end of the experiment period, to confirm the vagotomy surgery, the stomach food retention was evaluated for each group. The stomach was weighed and a ratio of stomach weight to total b.w. was taken. A confidence interval of 99% was employed to calculate the mean ratio for controls and its upper limit was used as a rejection criterion for vagotomy effectiveness (8).

Intraperitoneal insulin tolerance test (*ipITT*)

The *ipITT* were performed, as previously described in detail (32, 34).

Blood glucose and Insulinaemia

On the day following treatments, blood was collected from the tail tips of fed and 12 h-fasted rats and glucose levels were measured using a glucometer (“one touch” -Johnson & Johnson). Rats were then sacrificed (exposure to CO₂, followed by decapitation) and blood samples were stored at -20°C for measurement of insulin by RIA (39).

Isolation of islets and cumulative and dynamic secretion protocol

Islets were hand-picked after collagenase digestion of the pancreas, as previously described (10). For static incubation, five freshly-isolated islets were preincubated in 1 ml of Krebs-Ringer bicarbonate buffer containing 16 mM HEPES, 5.6 mM glucose, and 1% bovine serum albumin, pH 7.4, for 30 min at 37°C under an atmosphere of 95% O₂ and 5% CO₂. Islets were then incubated with 1 ml of fresh buffer containing glucose and cholinergic agonist or antagonist (as noted in legends of the figures). Sample aliquots for determination of insulin secretion were taken at 60-min time points and stored at -20°C for subsequent measurement of insulin content. For analysis of dynamic insulin secretion 20 freshly isolated islets were transferred to perfusion chambers and perfused with Krebs-bicarbonate buffer solution at a flow rate of 1ml/min for 55 min. Perfusion consisted of 4 consecutive periods: 30 min with 8.3 mM glucose; 10 min with glucose and 100 µM Cch; 10 min with glucose, Cch, and 100 µM atropine; and, finally, 5 min with glucose alone. The effluent was collected every min from the 20th min of perfusion and was stored at -20°C for insulin content.

Protein extraction and immunoblotting

Pools of islets were homogenized in ice-cold cell lysis buffer (Cell Signaling, MA, USA) using a cell homogenizer (Fisher Scientific, USA) according previous description (31,33). Protein concentration from total cell lysate was determined by the RCDC method (reducing agent compatible and detergent compatible assay), 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 five times using different samples (each sample consisting of islets obtained from one rat). After blocking at room temperature (RT) for 2 h in TBST/ 5% dry skimmed milk, membranes containing islet lysates were washed in TBST (3 x 7 min) and incubated overnight with primary antibodies in TBST/ 3% dry skimmed milk, at the dilutions recommended by the manufacturers. After washing in TBST (3 x 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, 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, MD, USA). β -actin was used as internal control.

Statistical Analysis

Results are expressed as the means \pm S.E.M. of the indicated number (n) of experiments. Analysis of variance (ANOVA) was used to compare unpaired groups, followed by Tukey *post test* for multiple comparisons of parametric data. The significance level adopted was $P < 0.05$.

RESULTS

DEX-induced insulin resistance and hyperinsulinemia in rats

Since dexamethasone treatment induced a marked decrease in insulin sensitivity (5, 20, 32) we verified whether vagotomy may alter the peripheral IR using the insulin tolerance test (*ipITT*). Figure 1A shows the constant of glucose decay (*Kitt*) after insulin challenge. The *Kitt* values were significantly reduced in SHAM DEX vs SHAM CTL rats ($P < 0.05$, $n = 8$). Similar results were observed when VAG DEX was compared to VAG CTL. Thus, vagotomy did not influence dexamethasone-induced peripheral IR. Fasting insulinaemia was significantly lower in VAG CTL (0.48 ± 0.01 ng/ml), compared with SHAM CTL (1.43 ± 0.09 ng/ml) rats ($P < 0.05$, $n = 8$). As expected, SHAM DEX displayed higher fasting insulinemia (15.27 ± 1.8 ng/ml), however, this adaptation was dampened in VAG DEX (2.74 ± 0.26 ng/ml) ($P < 0.05$, $n = 8$; Fig. 1B). Similar results were observed for insulinemia in fed rats. SHAM DEX fed insulinemia (25.18 ± 1.63 ng/ml) was significantly increased compared with SHAM CTL (5.49 ± 0.5 ng/ml), and decreased in VAG DEX (19.07 ± 0.9 ng/ml) compared with SHAM DEX ($P < 0.05$, $n = 8$; Fig. 1C). Fasting blood glucose values in both SHAM DEX and VAG DEX were higher than SHAM CTL and VAG CTL, respectively ($P < 0.05$, $n = 8$). However, VAG DEX blood glucose values (90.83 ± 3.58 mg/dl) were further increased when compared to those of SHAM DEX (79.66 ± 1.38 mg/dl) ($P < 0.05$, $n = 8$; Fig. 1D). Finally, VAG DEX rats exhibited reduced fed blood glucose levels compared to SHAM DEX, although higher than VAG CTL ($P < 0.05$, $n = 8$; Fig. 1E)

Cholinergic potentiation of GSIS in isolated islets

Figure 2 shows that islets isolated from DEX rats exhibit a higher glucose-induced cumulative insulin secretion, when compared to those isolated from CTL rats ($P < 0.05$, $n = 12$). The presence of Cch (100 μ M) potentiated glucose-induced insulin secretion by islets isolated from both CTL and DEX treated rats. However, the potentiation of insulin secretion by Cch was markedly increased in islets from DEX rats, when compared to islets from CTL rats ($P < 0.05$, $n = 12$). In order to assure that the Cch effect was due to activation of muscarinic receptors, we added atropine (100 μ M), a muscarinic antagonist, in our experimental design. Atropine significantly reduced the effect of Cch in both CTL and DEX islets ($P < 0.05$, $n = 12$). Experiments assessing the dynamics of insulin secretion revealed that the addition of 100 μ M Cch (10-20 min) resulted in increased insulin secretion in both CTL and DEX islets. The area-under-the-curve during the 30-40 min period was significantly higher in DEX than CTL islets ($P < 0.05$, $n = 4$; Fig. 2A and B), corroborating the finding of cumulative insulin secretion. After the introduction of atropine (40-50 min) to the perfusion medium, the potentiating effect of Cch was partially reduced. Finally, the secretion levels returned to their initial values when Cch and Atropine were withdrawn from the medium (50-55 min).

In order to assess the effects of *ex vivo* parasympathetic denervation, the cumulative insulin secretion in response to glucose, Cch and atropine was determined in islets isolated from vagotomized rats. Figure 3 shows that glucose-induced insulin secretion was increased in VAG DEX, compared to SHAM DEX ($P < 0.05$, $n = 12$). Vagotomy by itself resulted in an upregulation of insulin secretion upon combined stimulation with glucose and 100 μ M Cch (VAG CTL values were 6.2 ± 0.21 ng/ml *vs.* SHAM CTL values, which were 3.8 ± 0.22 ng/ml) ($P < 0.05$, $n = 12$). Additionally, insulin secretion by islets from VAG DEX was higher in response to Cch, compared to that secreted by islets from SHAM DEX ($P < 0.05$, $n = 12$). Atropine *per se* had no effect on insulin secretion induced by glucose, however this agent generally reduced the potentiation of the secretion of insulin by Cch. However, atropine inhibition of the Cch effect was more pronounced in VAG CTL and VAG DEX islets ($P < 0.05$, $n = 12$; Fig. 3).

Protein content of cholinergic components in isolated islets

The next set of experiments analyzed the content of proteins related to the islet cholinergic cascade. M3R content was 140% higher in islets from DEX rats than in those from CTL ($P < 0.05$, $n = 5$; Fig. 4), and 48% higher in islets from VAG DEX, compared with SHAM DEX ($P < 0.05$, $n = 5$, Fig. 5). PLC β 1 content was increased by 78% in DEX when compared to CTL islets ($P < 0.05$, $n = 5$, Figure 4). Vagotomy *per se* did not affect PLC β 1 protein expression. However, both SHAM DEX and VAG DEX islets presented higher PLC β 1 protein levels than their respective controls, SHAM CTL and SHAM DEX ($P < 0.05$, $n = 5$, Fig. 5). No differences in PKC α content were observed between DEX and CTL islets; however, phosphorylated PKC (pPKC) levels were 117% higher in DEX, compared with CTL islets ($P < 0.05$, $n = 5$, Fig. 4). PKC α protein content decreased by 68% in VAG CTL, compared with SHAM CTL ($P < 0.05$, $n = 5$, Fig. 5). The phosphorylated levels of PKC α increased by 49% and 81% in SHAM DEX and VAG DEX, compared to SHAM CTL and VAG CTL, respectively ($P < 0.05$, $n = 5$, Fig. 5).

DISCUSSION

IR is associated with the pathogenesis of *diabetes mellitus*, which constitutes one of the main threats to human health (5). The knowledge of the mechanisms that induce IR is important, since these may provide new perspectives for the development of new preventive strategies. It is well known that dexamethasone induces peripheral IR in rodents and humans (20, 32). In an insulin resistant state, alterations in the glucose metabolism of peripheral tissues such as liver, muscle and adipose tissue (as a result of the failure of these tissues to respond to insulin) occur (20). Peripheral IR increases plasma insulin levels as a consequence of oversecretion of insulin by pancreatic islets in an attempt to keep glycemia close to physiological ranges (7, 9, 25). We have formerly reported that rats treated with dexamethasone exhibit increased fasted and fed serum insulin as an indication of IR (31, 32). A previous study has reported that reduction of vagal inputs to the islets, by chemical blockade of the Ganglia in dexamethasone-treated IR humans, impaired arginine-induced insulin release, indicating a possible participation of the cholinergic mechanism in insulin oversecretion (2). In addition, a decrease in insulin response to glucose after vagotomy in dogs was observed (14). In this study, we investigated the contribution of the

cholinergic nervous system in dexamethasone-induced insulin resistance and hyperinsulinemia in rats, by withdrawing the vagus nerve.

VAG CTL and SHAM CTL exhibited similar Kitt values (Fig. 1A), indicating that vagotomy per se did not cause IR. VAG DEX and SHAM DEX rats also presented similar Kitt values, which indicate that vagotomy did not alter the ability of dexamethasone to induce IR (Fig. 1A). However, it is possible that an afferent vagal pathway links hepatic PPAR α to glucocorticoid-induced IR. This assumption is based on the observation that mice treated with dexamethasone for a long period of time, and submitted to hepatic vagal nerve sectioning, showed greater insulin responsiveness during the *ipITT* (24, 42). A possible explanation for this discrepancy may be related to the type and time of vagotomy and the time of dexamethasone treatment. Despite the lack of difference in IR between VAG DEX and SHAM DEX, vagotomy attenuated the enhancement of insulinemia induced by DEX treatment *in vivo*. This was represented by a marked reduction in fasting and fed serum insulin values in VAG DEX. These results indicate that the loss of cholinergic activity reduces insulinaemia, in agreement with previous reports suggesting that increased cholinergic activity accounts for hyperinsulinemia in obese insulin resistant mice and rats (3, 36). Further evidence associating increased cholinergic activity to higher insulin levels is shown by a study demonstrating that exposure to a high-carbohydrate (HC) milk diet during the suckling period results in permanent metabolic programming of hyperinsulinemia in HC rats that is reversed by vagotomy (23).

Fasting blood glucose levels were higher in VAG DEX, compared with SHAM DEX (Fig. 1D). Considering that IR and the consequent demand for insulin is similar in these groups, the lower insulinaemia in VAG DEX is probably due to the lack of parasympathetic activity. In the fed state, insulinaemia from VAG DEX is still lower than SHAM DEX but, as expected, much higher than in the fasting state. These rats showed lower glycemia in this condition (Fig. 1E). Whilst not easy to explain this apparent discrepancy, it should be kept in mind that rats with hepatic cholinergic denervation (IHCD) show a significant impairment in liver glycogen storage after feeding, as well as an impaired hepatic sensitivity to glucagon and, possibly, to insulin (45). In addition, in these rats, the absorptive period is reduced because vagotomy changes gastrointestinal motility, altering food intake and digestive behavior (8, 12).

Islets from DEX-treated rats exhibit an increased response to glucose both *in vivo* and *ex vivo*, when compared with control rats (32, 34). These alterations are accompanied by functional and

morphological changes in pancreatic beta cells (31). Whilst glucose is the primary stimulus for insulin secretion in β -cells, evidence exists that other secretagogues, including ACh, are involved in the regulation of GSIS (15). Here, we demonstrate that incubation of pancreatic islets from DEX rats with 100 μ M Cch, in the presence of 8.3 mM glucose, potentiated GSIS, indicating that hyperinsulinemia and increased insulin demand in this model are associated with increased parasympathetic activity. This finding is consistent with experiments with prolonged hyperglycemia in rats showing that hyperinsulinemia is dependent on increased parasympathetic activity (6), reversed by treatment with the cholinergic antagonist atropine. Furthermore, we have shown that exposure of DEX islets to atropine in the presence of Cch resulted in a reduction of cholinergic-induced insulin secretion, suggesting the mediation of M3R in this phenomenon, as proposed earlier (10). Additionally, islets isolated from DEX rats showed increased levels of M3R, compared with control islets (Fig. 4) (28).

After ACh binding to its receptor, different pathways of signal transductions are activated; one of the most important is the hydrolysis of phosphoinositides by phospholipase C (PLC) coupled to G-protein (43). Hydrolysis of PI results in formation of IP₃, which causes Ca^{2+} to exit from intracellular stores, raising its cytosolic concentration. This is accompanied by a rapid stimulation of insulin secretion (22). As cholinergic agonist activates PLC, diacylglycerol (DAG) is also formed (44), activating the Ca^{2+} -dependent protein kinase C (PKC) (30) and further enhancing GSIS. It is suggestive that PLC β 1 increased significantly in DEX, compared with CTL islets. No differences in PKC α content between DEX and CTL islets were observed (Fig. 4). In accordance, it has been postulated that activation of PKC is unlikely to be important, since overnight incubation of MIN6 cells with phorbol ester 12-o-tetradecanoylphorbol-13-acetate (TPA), which reduces the PKC activity in these cells, failed to affect acetylcholine-induced activation of the insulin granule movement (26). However, the levels of phosphorylated PKC α were significantly increased in DEX islets and this may justify the higher insulin secretion in these islets.

Interestingly, the experiments with isolated islets showed that the insulin secretion stimulated by glucose and Cch was higher in islets from vagotomized rats. This result indicates that vagotomy makes the islet more sensitive to these secretagogues, as already described by other groups (18). The observation that atropine inhibited Cch-induced insulin secretion to a great extent, in vagotomized rather than in control islets, suggests the mediation of the muscarinic receptors in

these events. This assumption is supported by the fact that M3R protein expression in vagotomized islets was significantly higher than their respective controls. Thus, vagotomy may lead to increased sensitivity to Cch in β -cells, underling the increased response to cholinergic agents.

In conclusion, dexamethasone-induced IR parallels a compensatory increase in insulinemia. This compensatory response is likely to result from activation of the parasympathetic neuron that sends signals to pancreatic islets, since vagotomy suppresses this response and islets isolated from DEX-treated rats express higher levels of M3R. An apparent paradoxical increase of insulin secretion was found in pancreatic islets from vagotomized DEX-treated rats. As judged by the increased M3R protein content in islets from VAG rats, the absence of vagus tonus makes the pancreatic islets more responsive to cholinergic stimulus, probably due to a compensatory mechanism that accounts for an increase in *ex vitro* secretory response. Thus, DEX-induced hyperinsulinemia in rats appears to be, at least in part, dependent upon the parasympathetic system.

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REFERENCES

1. Ahrén B. Autonomic regulation of islet hormone secretion implications for health disease. *Diabetologia* 43:393-410, 2000.
2. Arhén B. Evidence that autonomic mechanisms contribute to the adaptive increase in insulin secretion during dexamethasone-induced insulin resistance in humans. *Diabetologia* 51:1018-1024, 2008.
3. Ahrén B; Lundquist I. Modulation of basal insulin secretion in the obese hyperglycemic mouse. *Metabolism* 31:172-179, 1982.

4. Ahren B, Taborsky GJ Jr, Porte D Jr. Neuropeptidergic versus cholinergic and adrenergic regulation of islet hormone secretion. *Diabetologia* 29: 827–836, 1986.
5. Anderson JW, Kendall CW; Jenkins DJ. Importance of weight management in type 2 diabetes: review with meta-analysis of clinical studies. *J Am Coll Nutr* 22:331-339, 2003.
6. Balkan B, Dunning BE. Muscarinic stimulation maintains in vivo insulin secretion in response to glucose after prolonged hyperglycemia. *Am J Physiol Regul Integr Comp Physiol* 268:475–479, 1995.
7. Barbera M, Fierabracci V, Novelli M, Bombara M, Masiello P, Bergamini E, De Tata V. Dexamethasone-induced insulin resistance and pancreatic adaptive response in aging rats are not modified by oral vanadyl sulfate treatment. *Eur J Endocrinol* 145:799-806, 2001.
8. Bernstein, IL, Goehler, LE. Vagotomy produces learned food aversions in the rat *Behavior Neuroscience* 97:585-594, 1983.
9. Bonner-Weir S, Trent DF, Zmachinski CJ, Clore ET, Weir GC. Limited B cell regeneration in a B cell deficient rat model: studies with dexamethasone. *Metabolism* 9:914-918, 1981.
10. Boschero AC, Szpak-Glasmam M, Carneiro EM, Bordin S, Paul I, Rojas E, Atwater I. Oxometrine-m potentiation of glucose-induced insulin release from rat islets involves M3 muscarinic receptors. *Am J Physiol* 268:336-442, 1995.
11. Burém J, Liu HX, Jensen J, Eriksson JW. Dexamethasone impairs insulin signaling and glucose transport by depletion of insulin receptor substrate-1, phosphatidylinositol 3-kinase and protein kinase B in primary cultured rat adipocyte. *Eur J Endocrinol* 146:419-429, 2002.

12. Dixon KD, Williams FE, Wiggins RL, Pavelka J, Lucente J, Bellinger LL, Gietzen DW. Differential effects of selective vagotomy and tropisetron in aminoprivic feeding. *Am J Physiol Regul Integr Comp Physiol* 279:997–1009, 2000.
13. Edvell A, Lindström P. Vagotomy in young obese hyperglycemic mice: effects on syndrome development and islet proliferation. *Am J Physiol* 274:1034-1039, 1998.
14. Fischer U, Hommel H, Nowak W, von Dorsche HH, Sill U, Lippert H. Influence of vagotomy on glucose tolerance and on the response of plasma insulin and exocrine pancreatic function followed glucose load or food ingestion in dogs. *Acta Biol Med Ger* 35:1279–1291, 1976.
15. Gilon, P, Henquin, JC. Mechanisms and Physiological significance of the cholinergic control of pancreatic β -cell function. *Endocrine reviews* 22(5):565-604, 2001.
16. Holness MJ, Smith ND, Greenwood GK, Sugden MC. Interactive influences of peroxisome proliferator-activated receptor α activation and glucocorticoids on pancreatic beta cell compensation in insulin resistance induced by dietary saturated fat in the rat. *Diabetologia* 48: 2062-2068, 2005.
17. Hoogwerf B, Danese RD. Drug selection and the management of corticosteroid-related diabetes mellitus. *Rheum. Dis Clin North Am* 25:489-505, 1999.
18. Jarhult J, Åhrén B, Erichsen C, Lundquist I. Improved insulin release to carbachol after vagotomy in rats – an effect due to cholinergic supersensitivity of the β -cells? *Acta Physiol Scand.* 118:87-89, 1983.
19. Komatsu M, Schermerhorn T, Noda M, Straub SG, Aizawa T, Sharp GW. Augmentation of insulin release by glucose in the absence of extracellular Ca^{2+} : new insights into stimulus-secretion coupling. *Diabetes* 46: 1928–1938, 1997.

20. Larsson H, Ahrén B. Insulin resistant subjects lack islet adaptation to acute dexamethasone-induced reduction in insulin sensitivity. *Diabetologia* 42:936±943, 1999.
21. Larsson H, Ahrén B. Failure to adequately adapt reduced insulin sensitivity with increased insulin secretion in women with impaired glucose tolerance. *Diabetologia* 39:1099-1107, 1996.
22. Miller RE. Pancreatic neuroendocrinology: peripheral neural mechanisms in the regulation of the Islets of Langerhans. *Endocr Rev* 2: 471–494, 1981.
23. Mitrani P, Srinivasan M, Dodds C, Patel MS. Autonomic involvement in the permanent metabolic programming of hyperinsulinemia in the high-carbohydrate rat model. *Am J Physiol Endocrinol Metab.* 292: 1364–1377, 2007.
24. Mzrachi BC, Xiaozhong L, Yin L, Knutsen HR, Howard JM, Arends A JJ, DeSantis P, Coleman T, Semenkovich FC. An afferent vagal nerve pathway links hepatic PPAR α activation to glucocorticoid-induced insulin resistance and hypertension. *Cell metabolism* 5 (2): 91-102, 2007.
25. Nicod N, Giusti V, Besse C, Tappy L. Metabolic adaptations to dexamethasone-induced insulin resistance in healthy volunteers. *Obes Res* 11:625-631, 2003.
26. Niwa T, Matsukawa Y, Senda T, Nimura Y, Hidaka H, Niki I. Acetylcholine activates intracellular movement of insulin granules in pancreatic beta-cells via inositol triphosphate-dependent mobilization of intracellular Ca²⁺. *Diabetes* 47: 1699-1706 , 1998.
27. Novelli M, De Tata V, Bombara M, Lorenzini A, Masini M, Pollera M, Bergamini E, Masiello P. Insufficient adaptative capability of pancreatic endocrine function in dexamethasone-treated ageing rats. *J Endocrinol* 162:425-432, 1999.

28. Ostenson CG, Grill V. Evidence that hyperglycemia increases muscarinic binding in pancreatic islets of the rat. *Endocrinology* 121:1705–1710, 1987.
29. Pagano G, Cavallo PP, Cassader M, Bruno A, Ozzelo A, Masciola P, Dall'Omo AM, Imbimbo B. An in vivo and in vitro study of the mechanism of prednisone-induced insulin resistance in healthy subjects. *J Clin Invest* 72:1814-1820, 1983.
30. Persaud SJ, Jones PM, Howell SJ. Activation of protein kinase C is essential for sustained insulin secretion in response to cholinergic stimulation. *Biochim Biophys Acta* 1091:120-122, 1991.
31. Rafacho A, Cestari TM, Taboga SR, Boschero AC, Bosqueiro JR. High doses of dexamethasone induce increased beta-cell proliferation in pancreatic rat islets. *Am J Physiol Endocrinol Metab* 296(4): 681–690, 2009.
32. Rafacho A, Giozzet VAG, Boschero AC, Bosqueiro JR. Functional alterations in endocrine pancreas of rats with different degrees of dexamethasone-induced insulin resistance. *Pancreas* 6(3): 284-293, 2008.
33. Rafacho A, Ribeiro DL, Boschero AC, Taboga SR, Bosqueiro JR. Increased pancreatic islet mass is accompanied by activation of the IRS-2/AKT pathway and augmented cyclin D2 protein levels in insulin resistant rats. *Int J Exp Pathol* 89(4):264-275, 2008.
34. Rafacho A, Roma LP, Taboga SR, Boschero AC, Bosqueiro JR. Dexamethasone induced insulin resistance is associate with increase of connexin 36mRNA and protein expression in pancreatic rat islets. *Can J Physiol Pharmacol* 85:881-885, 2007.
35. Rasmussen H, Zawulich KC, Ganesan S, Calle R, Zawulich WS. Physiology and pathophysiology of insulin secretion. *Diabetes Care* 13:655–666, 1990.

36. Rohner-Jeanrenaud F, Hochstrasser AC, Jeanrenaud B. Hiperinsulinemia of preobese and obese fa/fa rats is partly vagus nerve mediated. *Am J Physiol* 244:317-322, 1983.
37. Ruzzin J, Wagman AS, Jensen J. Glucocorticoid-induced insulin resistance in skeletal muscles: defects in insulin signaling and the effects of a selective glycogen synthase kinase-3 inhibition. *Diabetologia* 48:2119-2130, 2005.
38. Saad MJ, Folli F, Kahn JA, Kahn CR. Modulation of insulin receptor, insulin receptor substrate-1, and phosphatidylinositol 3-kinase in liver and muscle of dexamethasone-treated rats. *J Clin Invest* 92: 2065-2072, 1993.
39. Scott AM, Atwater I, Rojas E. A method for the simultaneous measurement of insulin release and B cell membrane potential in single mouse islets of Langerhans. *Diabetologia* 21:470-475, 1981.
40. Severino C, Brizzi P, Solinas A, Secchi G, Maioli M, Tonolo G. Low-dose dexamethasone in the rat. A model to study insulin resistance. *Am J Physiol* 283:367-373, 2002.
41. Shino A, Matsuo T, Iwatsuka I, Suzuoki Z. Estructural changes of pancreatic islets in genetically obese rats. *Diabetologia* 9: 413-421, 1973.
42. Uno K, Katagiri H, Yamada T, Ishigaki Y, Ogihara T, Imai J, Hasegawa Y, Gao J, Kaneko K, Iwasaki H, Ishihara H, Sasano H, Inukai K, Mizuguchi H, Asano T, Shiota M, Nakazato M, Oka Y. Neuronal pathway from the liver modulates energy expenditure and systemic insulin sensitivity. *Science* 312: 1656-1659, 2006.
43. Verspohl EJ, Herrmann K. Involvement of G proteins in the effect of carbachol and cholecystokinin in rat pancreatic islets. *Am J Physiol* 271:65-72 , 1996.

44. Weng L, Davies M, Ashcroft SJ. Effects of cholinergic agonists on diacylglycerol and intracellular calcium levels in pancreatic beta-cells. *Cell Signal* 5: 777-786, 1993.
45. Xue C, Aspelund G, Sritharan KC, Wang JP, Slezak LA, Andersen DK. Isolated hepatic cholinergic denervation impairs glucose and glycogen metabolism. *J Surg Res.* 1;90 (1):19-25, 2000.

FIGURE LEGENDS

Figure 1. *DEX-induced insulin resistance and hyperinsulinemia in rats.* As accessed by the glucose decay values (Kitt) obtained from the intraperitoneal insulin tolerance test, administration of DEX for five days significantly decreased insulin sensitivity in SHAM DEX *vs* SHAM CTL and this significant decay was repeated in VAG CTL and VAG DEX rats (**A**). Interestingly, fasting insulinaemia decreased by 67% in VAG CTL *vs* SHAM CTL and 90% in VAG DEX *vs* SHAM DEX (**B**). The same pattern observed during fasting was also noted to fed insulinaemia with a decrease of 27% in VAG CTL *vs* SHAM CTL and 25% in VAG DEX *vs* SHAM DEX (**C**). Fasted blood glucose increased by 14% in VAG DEX *vs* SHAM DEX (**D**) and decreased in VAG DEX by 62% *vs* SHAM DEX (**E**). Values are means \pm S.E.M. * significantly different *vs* saline treated, # significantly different *vs* sham operated. $n = 8$ for all. $P < 0.05$, ANOVA with *post test* Tukey.

Figure 2. *Cholinergic potentiation of GSIS in isolated islets.* In (**A**) the cumulative GSIS in isolated DEX islets in response to cholinergic agonist and antagonist, in the presence of 8.3mM glucose. Cch (100 μ M) treatment resulted in the potentiation of insulin secretion in CTL and DEX islets, when compared with the insulin secretion in islets incubated with glucose alone. Observe the higher insulin secretion in islets from DEX rats when stimulated by Cch than CTL islets. * significantly different *vs* 8.3mM glucose alone; # significantly different *vs* CCh 100 μ M; ^a significantly different *vs* CTL (8.3 mM glucose); ^b significantly different *vs* CTL (100 μ M Cch). In (**B**) the GSIS by perfused DEX islets in response to cholinergic agonist and antagonist in the presence of 8.3mM glucose and in (**C**) the area-under-the-curve (AUC) data revealing an

increased insulin response to 100 μ M Cch vs glucose alone (Cch 0 μ M). * significantly different vs CTL. Values are means \pm S.E.M. $n = 12$ for A and 4 for B (obtained from 2 separate days of experiment). $P < 0.05$, ANOVA with *post test* Tukey.

Figure 3 *GSIS by SHAM and VAG isolated islets.* Insulin response to cholinergic agonist and antagonist in the presence of 8.3mM glucose in all groups. β -cells release more insulin in response to the parasympathomimetic agent, carbachol, if the pancreas is deprived of its normal vagal innervation. Values are means \pm S.E.M. * significantly different vs 8.3mM glucose alone; # significantly different vs CCh 100 μ M; ^a significantly different vs SHAM CTL (8.3mM glucose), ^b significantly different vs SHAM CTL (100 μ M CCh), ^c significantly different vs VAG CTL (8.3mM glucose), ^d significantly different vs SHAM DEX (8.3mM glucose), ^e significantly different vs VAG CTL (100 μ M CCh), ^f significantly different vs SHAM DEX (100 μ M CCh). $n = 12$ (obtained from 2 separate days of experiment), $P < 0.05$, ANOVA with *post test* Tukey.

Figure 4 *Cholinergic protein content in DEX islets.* Measurements of M3R (Muscarinic receptor type 3), PLC β 1 (Phospholipase C β 1), PKC α (protein kinase C α), and pPKC (phosphorylated protein kinase C α) protein content. The figures are representative immunoblots performed at least five times on separate islet extracts. Below the panel, a representative control blot for β -actin is shown. Values are means \pm S.E.M. * significantly different vs CTL. $P < 0.05$. ANOVA with *post test* Tukey.

Figure 5 *Cholinergic protein content in SHAM and VAG islets.* Measurements of M3R (Muscarinic receptor type 3), PLC β 1 (Phospholipase C β 1), PKC α (protein kinase C α), and pPKC (phosphorylated protein kinase C α) content. The figures are representative immunoblots

performed at least five times on separate islet extracts. Below the panel, a representative control blot for β -actin is shown. Values are means \pm SE. * significantly different vs saline treated, # significantly different vs SHAM operated. $P < 0.05$. ANOVA with *post test* Tukey.

FIGURES

Figure 1

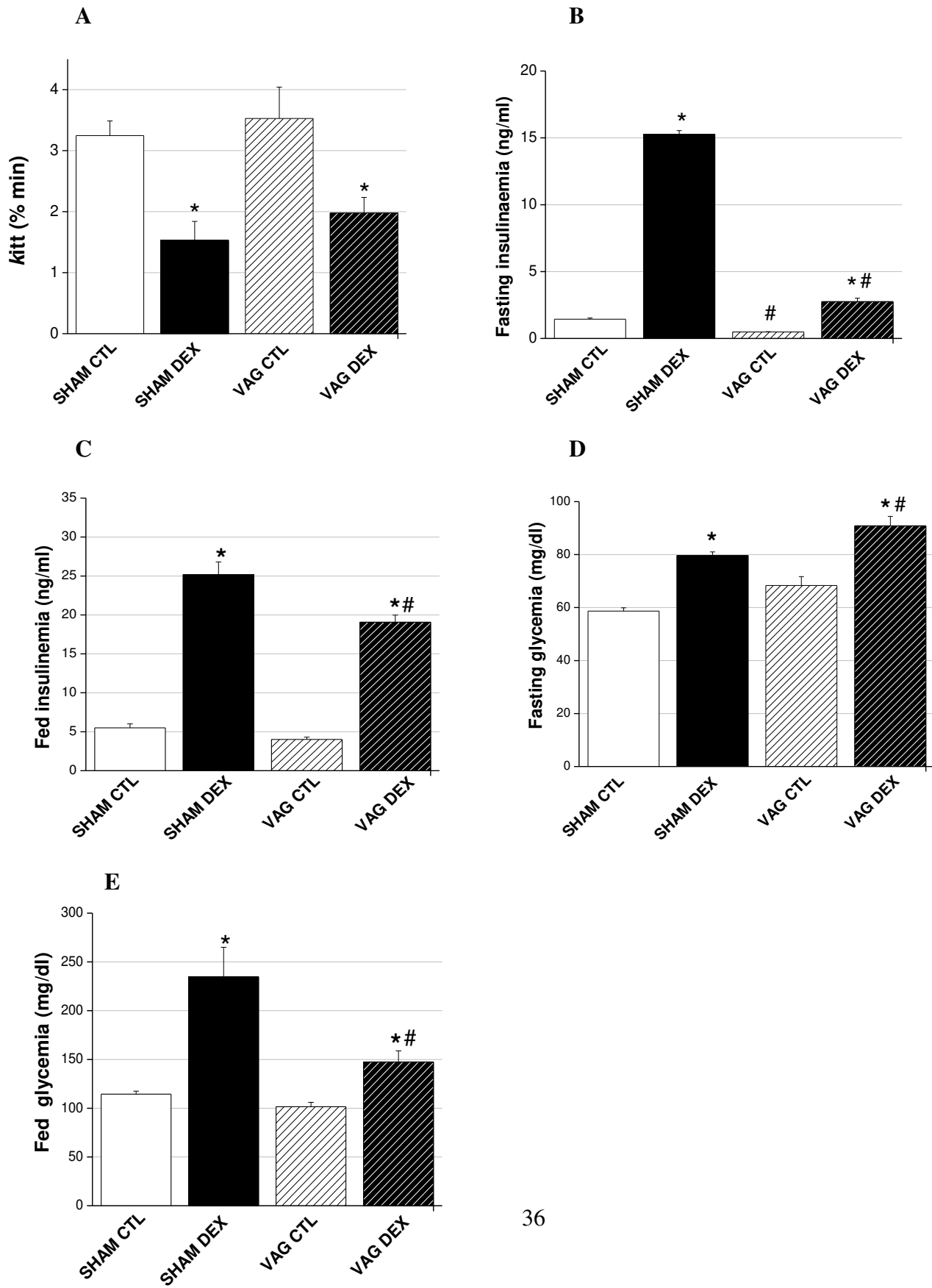


Figure 2

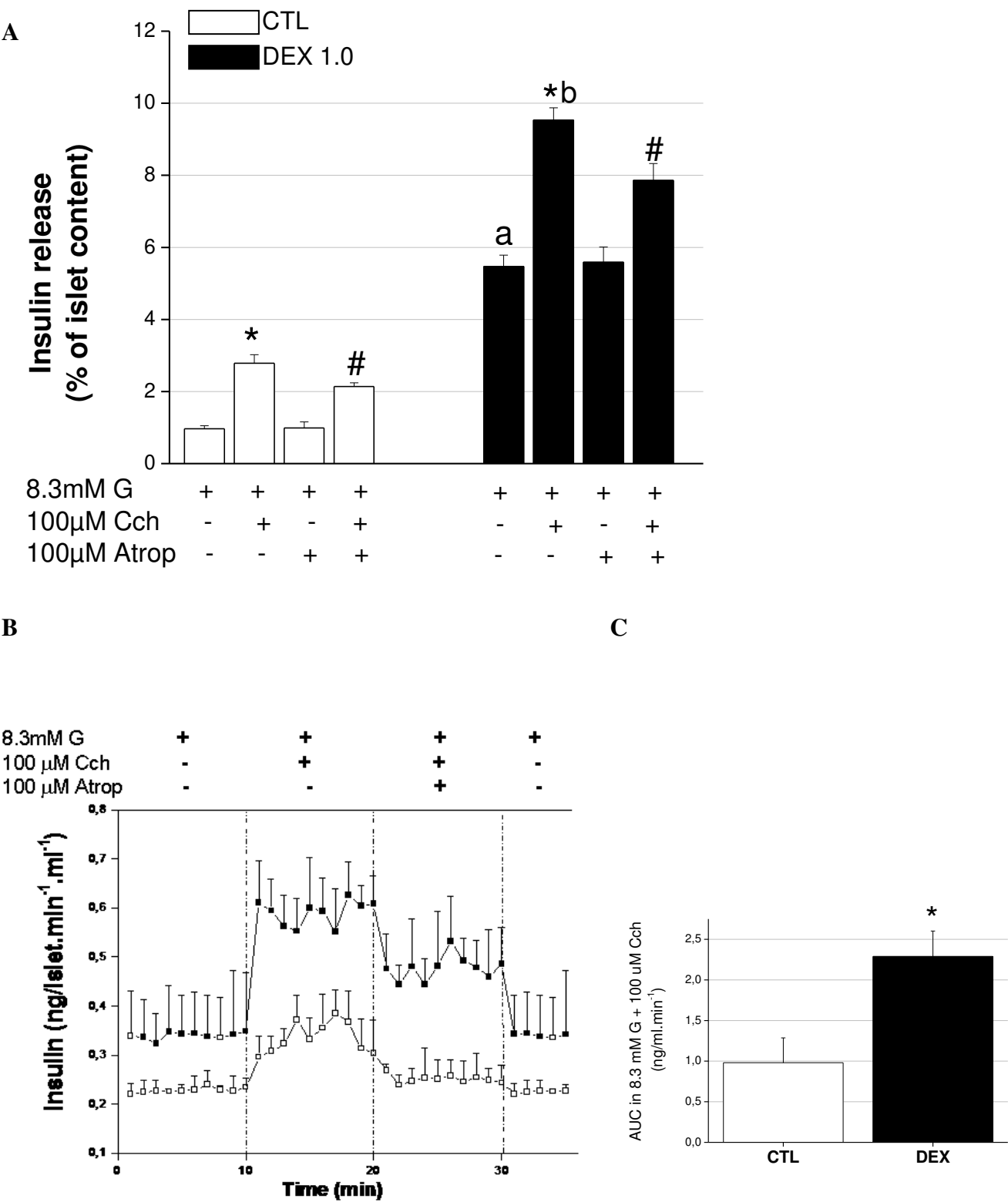


Figure 3

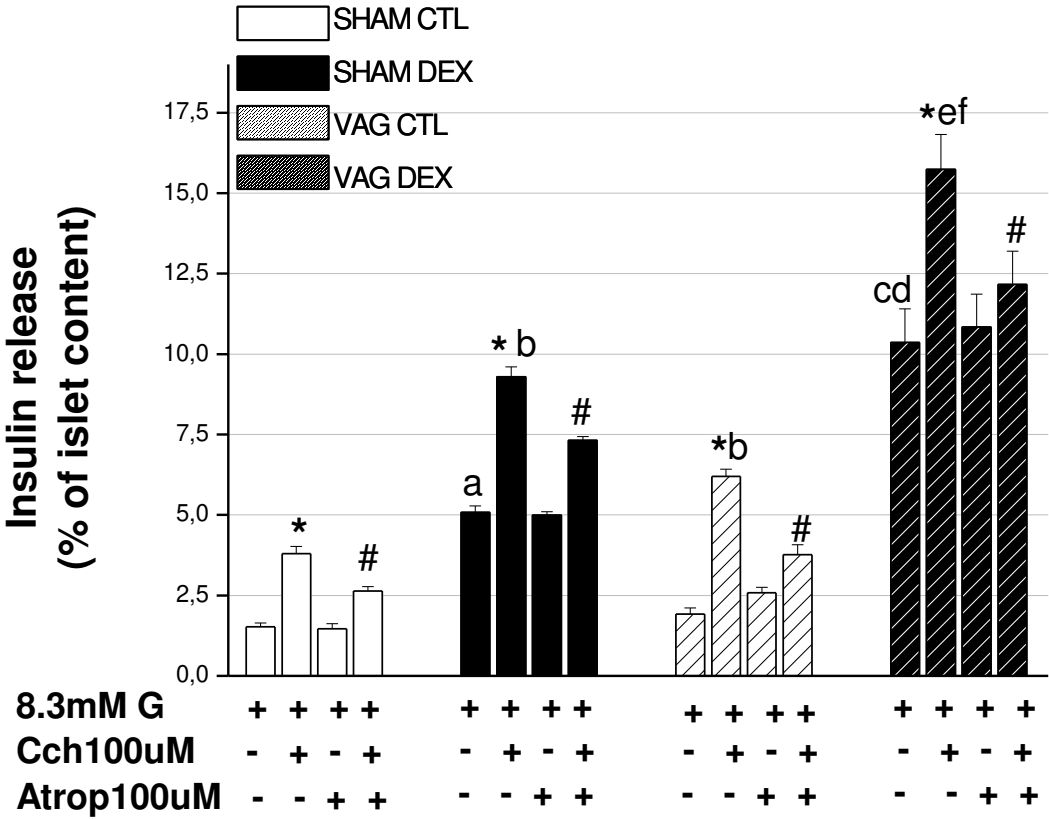


Figure 4

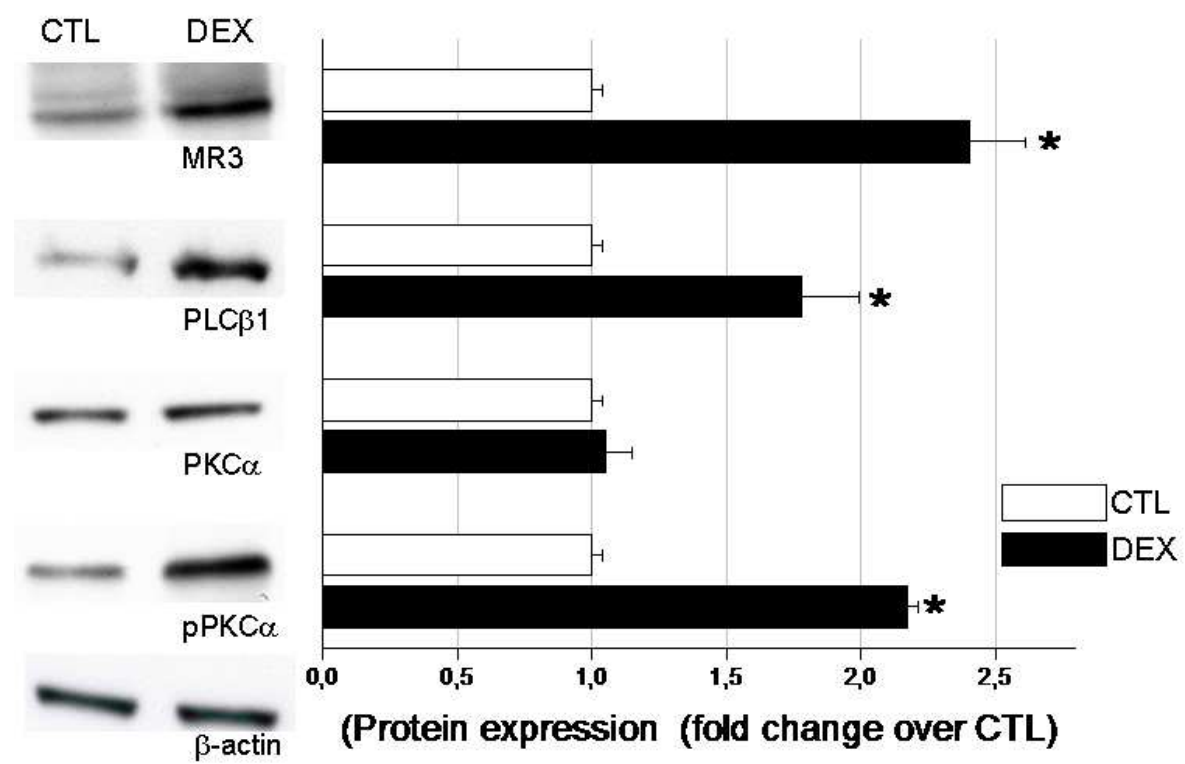
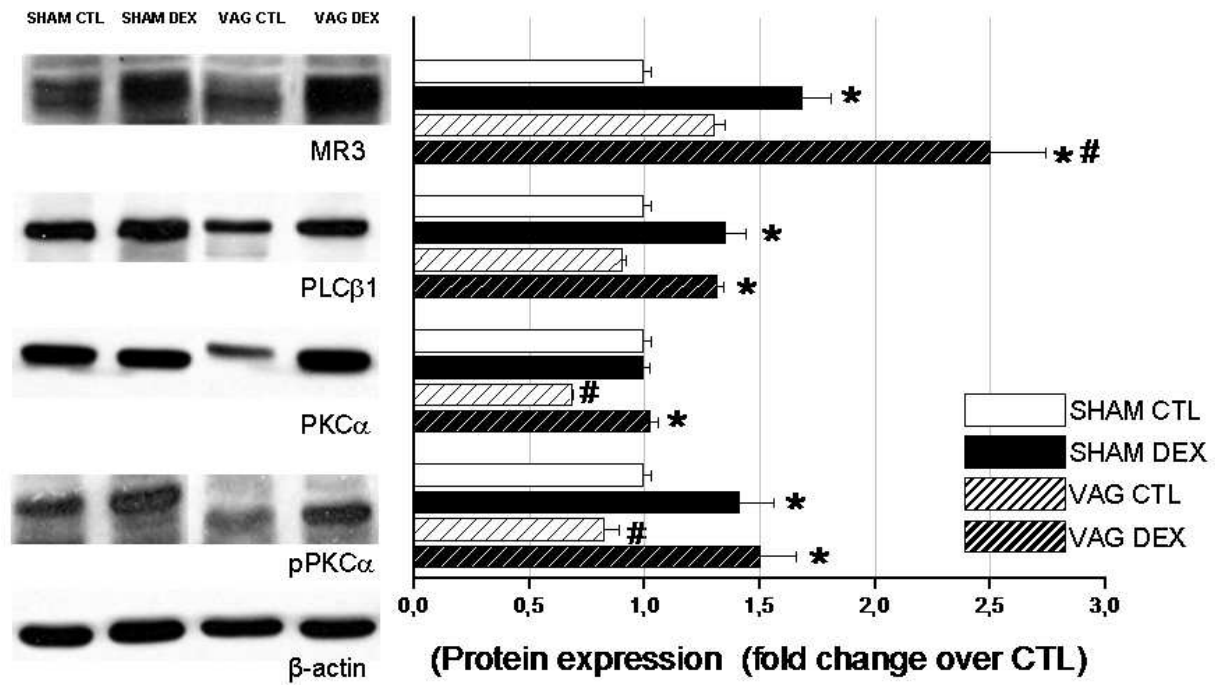


Figure 5



Os resultados obtidos nesse trabalho nos levam a concluir que:

- o tratamento com DEX por 5 dias consecutivos induz RI, correlacionando-se positivamente com os níveis circulantes de insulina;
- o aumento dos níveis de insulina sérica observado nos animais DEX pode ter contribuição de mecanismos independentes do metabolismo como o sistema nervoso parassimpático;
- ilhotas de animais CTL DEX são mais responsivas ao agonista colinérgico (Cch) e estas mudanças na regulação autonômica são apoiadas por aumentos no conteúdo de proteínas sinalizadoras da via parassimpática;
- a ausência do tônus vagal torna as ilhotas VAG CTL e VAG DEX mais responsáveis ao agonista colinérgico (Cch) provavelmente por um mecanismo de *feedback*, apoiado pelos aumentos conteúdo de proteínas sinalizadoras da via parassimpática;
- vagotomia resultou em atenuação dos efeitos da Dex nos níveis de insulina *prandial* e *pos-prandial*, Entretanto a vagotomia *per se* não influencia na capacidade da Dex induzir RPI;

Em conclusão, ratos com RI induzida pelo tratamento com dexametasona, exibem níveis elevados de insulina circulante, que está associado com uma resposta aumentada ao estímulo colinérgico pelas ilhotas e maior conteúdo de proteína MR3. Estas mudanças podem funcionar para preservar a capacidade aumentada de secreção de insulina pelas células β , em animais DEX, para manter a euglicemia. A vagotomia reduziu a hiperinsulinemia em ratos tratados com DEX tanto no estado de jejum como alimentado, confirmando a participação do SNA colinérgico na hipersecreção de insulina induzida pelo tratamento. A julgar pelo aumento do conteúdo de proteína MR3 em ilhotas de animais vagotomizados, a ausência do tônus vagal torna as ilhotas

pancreáticas mais responsivas ao estímulo colinérgico, provavelmente devido a um mecanismo compensatório. Entretanto estudos adicionais são necessários para determinar a extensão do envolvimento do SNA, incluindo a contribuição do sistema nervoso adrenérgico e a taxa de atividade deste sistema. Estes estudos ajudarão nosso entendimento sobre a crescente epidemia do Diabetes tipo 2.

V-REFERÊNCIAS BIBLIOGRÁFICAS

Ahrén B. (2000) *Autonomic regulation of islet hormone secretion implications for health disease.* **Diabetologia** 43:393-410

Ahrén B, Karlsson S, Lindskog S. (1990) *Cholinergic regulation of the endocrine pancreas.* In. Aquilonius S, Gilberg PG (eds) *Progress in Brain research, Cholinergic Neurotransmission.* **Elsevier Amsterdam** 84:209-218

Ahrén B, Lundquist I. (1981) *Effects of selective and nonselective β -adrenergic agents on insulin secretion in vivo.* **Eur J Pharmacol.** 71:93-104

Ahrén B, Lundquist I. (1982) *Modulation of basal insulin secretion in the obese, hyperglycemic mouse.* **Metabolism.** 31:172-179

Ahrén B, Pacini G. (2005) *Islet adaptation to insulin resistance: mechanism and implications for intervention.* **Diabetes Obes. Metab.** 7:2-8

Ahren B, Sundkvist G, Mulder H, Sundler F. (1996) *Blockade of muscarinic transmission increases the frequency of diabetes after low-dose alloxan challenge in the mouse.* **Diabetologia** 39:383-390

Ahren B, Taborsky GJ, Jr, Porte D Jr. (1986) *Neuropeptidergic versus cholinergic and adrenergic regulation of islet hormone secretion.* **Diabetologia** 29:827-836

Amatruda JM, Livingston JN, Lockwood DH. (1985) *Cellular Mechanism in selected states of insulin resistance: human obesity, glucocorticoid excess and chronic renal failure.* **Diabetes Metabolism Rev.** 3:296-317

Anderson JW, Kendall CW, Jenkins DJ. (2003) *Importance of weight management in type 2 diabetes: review with meta-analysis of clinical studies.* **J. Am. Coll. Nutr.** 22:331-339

Andrews RC, Walker BR. (1999) Glucocorticoids and insulin resistance: old hormones, new targets. **Clin Sci.** 96:513-523

Balbo SL, Bonfleur ML, Carneiro EM, Amaral ME, Filiputti E, Mathias PC. (2002) *Parasympathetic activity changes insulin response to glucose and neurotransmitters.* **Diabetes Metab** 28:3:13–317

Balkan B, Dunning BE. (1995) *Muscarinic stimulation maintains in vivo insulin secretion in response to glucose after prolonged hyperglycemia.* **Am J Physiol Regul Integr Comp Physiol.** 268: 475–479

Barbera M, Fierabracci V, Novelli M, Bombara M, Masiello P, Bergamini E, De Tata V. (2001) *Dexamethasone-induced insulin resistance and pancreatic adaptive response in aging rats are not modified by oral vanadyl sulfate treatment.* **Eur. J. Endocrinol.** 145:799-806

Bloom SR, Edwards AV. (1985) *Effects of certain metabolites on pancreatic endocrine response to stimulation of the vagus nerves in conscious calves.* **J Physiol.** 362:303-310

Bonner-Weir S, Trent DF, Weir GC. (1983) *Partial pancreatectomy in the rat and subsequent defect in glucose induced insulin secretion.* **J. Clin. Invest.** 71:1544-1553

Bonner-Weir S, Trent DF, Zmachinski CJ, Clore ET, Weir GC. (1981) *Limited B cell regeneration in a B cell deficient rat model: studies with dexamethasone.* **Metabolism.** 9:914-918

Boschero AC. (1996) *Acoplamento da estimulação-secreção de insulina pelas células β pancreáticas.* **Arq. Bras. Endocrinol. Metab.** 40:149-155

Bratanova-Tochkova TK, Cheng H, Daniel S, Gunawardana S, Liu YJ, Mulvaney-Musa J, Schermerhorn T, Straub SG, Yajima H, Sharp GW. (2002) *Triggering and augmentation mechanisms, granule pools, and biphasic insulin secretion.* **Diabetes** 51(1) 83–90

Brunnicardi FC, Shavelle DM, Adersen DK. (1995) *Neural regulation of the endocrine pancreas*. **Int J Pancreatol** 18:177-195

Burém J, Liu HX, Jensen J, Eriksson JW. (2002) *Dexamethasone impairs insulin signaling and glucose transport by depletion of insulin receptor substrate-1, phosphatidylinositol 3-kinase and protein kinase B in primary cultured rat adipocyte*. **Eur J. Endocrinol.** 146:419-429

Burén J, Lai YC, Lundgren M, Eriksson JW, Jensen J. (2008) *Insulin action and signalling in fat and muscle from dexamethasone-treated rats*. **Arch Biochem Biophys** 474:91-101.

Cerasi E, Nesher R, Gadot M, Gross D, Kaiser N. (1995) *Insulin secretion in obese and non-obese NIDDM*. **Diabetes Res Clin Pract** 28:27-37

Chan SL, Perrett CW Morgan NG. (1997) *Differential expression of alpha α 2-adrenoceptor subtypes in purified rat pancreatic islet α - and β -cells*. **Cell Signal.** 9:71-78

Cnop M, Vidal J, Hull RL, Utzschneider KM, Carr DB, Schraw T, Scherer PE, Boyko EJ, Fujimoto WY, Kahn SE. (2007) *Progressive loss of beta-cell function leads to worsening glucose tolerance in first-degree relatives of subjects with type 2 diabetes*. **Diabetes Care** 30:677-682

Cooper DMF, Mons N, Karpen JW. (1995) *Adenylyl cyclases and the interaction between calcium and cAMP signaling*. **Nature.** 374:421-424

Delaunay F, Khan A, Cintra A, Davani B, Ling ZC, Andersson A, Ostenson CG, Gustafsson JA, Enfendic S, Okret S. (1997) *Pancreatic β -cells are important for the diabetogenic effects of glucocorticoids*. **J. Clin. Invest.** 100(8):2094-2098

Del Rio G, Procopio M, Bondi M, Marrama P, Menozzi R, Oleandri SE, Grottoli S, Maccario M, Velardo A, Ghigo E. (1997) *Cholinergic enhancement by pyridostigmine increases the*

insulin response to glucose load in obese patients but not in normal subjects. Int J Obes. 21:1111-1114

Dixon KD, Williams FE, Wiggins RL, Pavelka J, Lucente J, Bellinger LL, Gietzen DW. (2000) *Differential effects of selective vagotomy and tropisetron in aminoprivic feeding. Am J Physiol Regul Integr Comp Physiol* 279:997–1009

Dolz M, Bailbe D, Giroix MH, Calderari S, Gangnerau MN, Serradas P, Rickenbach K, Irminger JC, Portha B. (2005) *Restitution of defective glucose-stimulated insulin secretion in diabetic GK rat by acetylcholine uncovers paradoxical stimulatory effect of beta-cell muscarinic receptor activation on cAMP production. Diabetes* 54: 3229–3237

Doyle EM, Egan M J. (2003) *Pharmacological Agents That directly Modulate Insulin Secretion.* The American Society for pharmacology and Experimental Therapeutic. 5:105 – 131

Edvell A, Lindström P. (1998) *Vagotomy in young obese hyperglycemic mice: effects on syndrome development and islet proliferation. Am J Physiol.* 274:1034-1039

Efendic S, Luft R, Wajngot A. (1984) *Aspects of the patogenesis of type 2 diabetes. Endocrinol Reviews* 5:395-410

Escriva F, Rodriguez C, Cacho J, Alvarez C, Portha B, Pascual-Leone AM. (1992) *Glucose utilization and insulin action in adult rats submited to prolonged food restriction. Am. J. Physiol.* 263: 1-7

Eizirik DL, Mandrup-Poulsen T. (2001) *A choice of death-the- signal-transduction of immune-mediated beta-cell apoptosis. Diabetologia* 44:2115–2133.

Festa A, Williams K, D'Agostino R Jr, Wagenknecht LE, Haffner SM. (2006) *The natural course of beta-cell function in nondiabetic and diabetic individuals: the Insulin Resistance Atherosclerosis Study. Diabetes* 55:1114–1120.

Ferreira F, Filiputti E, Arantes VC, Stoppiglia LF, Araújo EP, Delghingaro-Augusto V, Latorraca MQ, Toyama MH, Boschero AC, Carneiro EM. (2003) *Decreased cholinergic stimulation of insulin secretion by islets from rats fed a low protein diet is associated with reduced protein kinase c alpha expression.* **Journal of Nutrition** 133:695-699

Filipsson K, Karlsson S, Ahren B. (1998) *Evidence for contribution by increased cytoplasmic Na⁺ to the insulinotropic action of PACAP38 in HIT-T15 cells.* **J Biol Chem.** 273:32602-32607

Flatt PR. (1991) *Textbook of Diabetes*, [J.C. Pickup and G. Williams, editors] Oxford: Blackwell, 2nd ed pp.9.1-9

Gembal M, Detimary P, Gilon P, Gao Z-Y, Henquin JC. (1993) *Mechanisms by which glucose can control insulin release independently from its action on adenosine triphosphate-sensitive K⁺ channels in mouse B cells.* **J Clin Invest.** 91:871–880

Gilon P, Henquin JC. (2001) *Mechanisms and Physiological significance of the cholinergic control of pancreatic β -cell function.* **Endocrine reviews** 22(5):565-604

Godfrey DA, Matschinnsky FM. (1975) *Enzymes of Cholinergic system in islets of Langerhans.* **J Histochem Cytochem.** 23:645-651

Gremlich S, Roduit R, Thorens B. (1997) *Dexamethasone induces posttranslational degradation of GLUT2 and inhibition of insulin secretion in isolated pancreatic B cells. Comparison with the effects of fatty acids.* **J Biol Chem.** 272:3216-3222

Grill V, Cerasi E. (1973) *Activation by glucose of adenyl cyclase in pancreatic islets of the rat.* **FEBS Lett.** 33:311–314

Grill V, Cerasi E. (1974) *Stimulation by D-glucose of cyclic adenosine 3',5'-monophosphate accumulation and insulin release in isolated pancreatic islets of the rat.* **J Biol Chem.** 249:4196–4201

Henquin J-C, Meissner HP. (1984) *The ionic, electrical, and secretory effects of endogenous cyclic adenosine monophosphate in mouse pancreatic B cells: studies with forskolin.* **Endocrinology** 115:1125–1134

Holness M J, Smith ND, Greenwood GK, Sugden MC. (2005) *Interactive influences of peroxisome proliferator-activated receptor α activation and glucocorticoids on pancreatic beta cell compensation in insulin resistance induced by dietary saturated fat in the rat.* **Diabetologia** 48:2062-2068

Hoogwerf B, Danese RD. (1999) *Drug selection and the management of corticosteroid-related diabetes mellitus.* **Rheum. Dis. Clin. North. Am.** 25:489-505

Huang PK. (1989) *The mechanism of protein kinase C activation.* **Trend. Biochem. Sci.** 12(11):425-31

Jeong IK, Oh SH, Kim BJ, Chung JH, Min YK, Lee MS, Lee MK, Kim KW. (2001) *The effects of dexamethasone on insulin release and biosynthesis are dependent on the dose and duration of treatment.* **Diab. Reserc. Clin. Pract.** 51:163-17

Jones SV. (1993) *Muscarinic receptor subtypes: modulation of ion channels.* **Life science** 52:457-464

Kahn SE, Montgomery B, Howell W, Ligueros-Saylan M, Hsu CH, Devineni D, McLeod JF, Horowitz A, Foley JE. (2001) *Importance of early phase insulin secretion to intravenous glucose tolerance in subjects with type 2 diabetes mellitus.* **J Clin Endocrinol Metab.** 86:4047–4058

Kahn SE, Prigeon RL, Mcculloch DK, Boyko EJ, Bergman RN, Schwartz MW, Neifing JL, Ward WK, Beard JC, Palmer JP, *et al.* (1993) *Quantification of the relationship between insulin sensitivity and beta-cell function in human subjects. Evidence for a hyperbolic function.* **Diabetes** 42:1663-1672

Kanda F, Takatanik Okuda S, Matsushita T, Chihara K. (1999) *Preventive effects of insulin-like growth factor on steroid-induced muscle atrophy.* **Muscle Nerve** 22:213-217

Karlsson S, Ahren B. (1999) *Cytosolic Ca^{2+} oscillations by gastrin releasing peptide in single HIT-T15 cells.* **Peptides** 20:579-587

Kaway A, Kuzuya N. (1977) *On the role of glucocorticoid in glucose-induced insulin secretion.* **Horm. Metab. Res.** 9(5): 361-365

Kasuga M. (2006) *Insulin resistance and pancreatic beta cell failure.* **J Clin Invest.** 116:1756–1760

Kelley GG, Zawulich WS, Zawulich KC. (1995) *Synergistic interaction of glucose and neurohormonal agonist to stimulate islet phosphoinositide hydrolysis.* **Am. Journal Physiology** 269:575-582

Kohnert KD, Myrsén-Axcrona U, Hehmke B, Klöting I, Sundler F, Ahrén B. (1999) *Islet neuronal abnormalities associated with impaired insulin secretion in type 2 diabetes in the Chinese hamster.* **Regul Pept.** 82:71-79

Kurose T, Seino Y, Nishi S, Tsuji K, Taminato T, Tsuda K, Imura H. (1990) *Mechanism of sympathetic neural regulation of insulin, somatostatin, and glucagon secretion.* **Am j Physiol.** 258:220-227

Lacey RJ, Berrow NS, Scarpello JH, Morgan NG. (1991) *Selective stimulation of glucagon secretion by beta 2- adrenoceptors in isolated islets of Langerhans of the rat.* **Br J Pharmacol.** 103:1824-1828

Lacy PE, Kostianovsky M. (1967) *Method for the isolation of intact islets of langerhans from the rat pancreas.* **Diabetes** 16:35-39

Lacy, PE, Kostianovsky M. (1967) *Method for the isolation of intact islets of langerhans from the rat pancreas.* **Diabetes** 16: 35-39

Larsson H, Ahrén B. (1996) *Failure to adequately adapt reduced insulin sensitivity with increased insulin secretion in women with impaired glucose tolerance.* **Diabetologia** 39: 1099-1107

Larsson H, Ahrén B. (1999) *Insulin resistant subjects lack islet adaptation to acute dexamethasone-induced reduction in insulin sensitivity.* **Diabetologia** 42: 936-943

Laury MC, Takao F, Bailbe D, Penicaud L, Portha B, Picon L, Ktorza A. (1991) *Differential effects of prolonged hyperglycemia on in vivo and in vitro insulin secretion in rats.* **Endocrinology** 128:2526–2533

Leiser M, Fleischer N. (1996) *cAMP-dependent phosphorylation of the cardiac-type $\alpha 1$ subunit of the voltage-dependent Ca^{2+} channel in a murine pancreatic bcell line.* **Diabetes** 45:1412–1418

Leon-Quinto T, Magnan C, Portha B (1998) *Altered activity of the autonomous nervous system as a determinant of the impaired beta-cell secretory response after protein-energy restriction in the rat.* **Endocrinology** 139:3382–3389

Malaisse WJ, Malaisse-Lagae F (1984) *The role of cyclic AMP in insulin release.* **Experientia** 40:1068–1075

Michael MD, Kulkarni RN, Postic C, Previs SF, Shulman GI, Magnuson MA, Kahn CR. (2000) *Loss of insulin signaling in hepatocytes leads to severe insulin resistance and progressive hepatic dysfunction.* **Mol Cell.** 6:87–94

Mitrani P, Srinivasan M, Dodds C, Patel MS. (2007) *Autonomic involvement in the permanent metabolic programming of hyperinsulinemia in the high-carbohydrate rat model* **Am J Physiol Endocrinol Metab.** 292:1364–1377 (A)

Mitrani P, Srinivasan M, Dodds C, Patel MS. (2007) *Role of the autonomic nervous system in the development of hyperinsulinemia by high-carbohydrate formula feeding to neonatal rats.* **Am J Physiol Endocrinol Metab.** 292:1069–1078 (B)

Miyake K, Ogawa W, Matsumoto M, Nakamura T, Sakaue H, Kasuga M. (2002) *Hyperinsulinemia, glucose intolerance, and dyslipidemia induced by acute inhibition of phosphoinositide 3-kinase signaling in the liver.* **J Clin Invest.** 110:1483–1491

Myojin T, Kitamura N, Hondo E, Baltazar E.T, Pearson GT, Yamada J. (2000) *Immunohistochemical localization of neuropeptides in bovine pancreas.* **Anat Histol Embryol.** 29:167-172

Nakaki T, Nakadate T, Ishii K, Kato R. (1981) *Postsynaptic α_2 -adrenergic receptors in isolated rat islets of Langerhans: inhibition of insulin release and cyclic 3',5'-adenosine monophosphate accumulation.* **J Pharmacol Exp Ther.** 216:607-612

Nicod N, Giusti V, Besse C, Tappy L. (2003) *Metabolic adaptations to dexamethasone-induced insulin resistance in healthy volunteers.* **Obes Res.** 11:625-631

Nilsson T, Arkhammar P, Rorsman P, Berggren PO. (1988) *Inhibition of glucose-stimulated insulin release by α_2 adrenoceptor activation is paralleled by both a repolarization and a reduction in cytoplasmic free Ca^{2+} concentration.* **J Biol Chem.** 263: 1855-1860

Niwa T, Matsukawa Y, Senda T, Nimura Y, Hidaka H, Niki I. (1998) *Acetylcholine activates intracellular movement of insulin granules in pancreatic beta-cells via inositol triphosphate-dependent mobilization of intracellular Ca^{2+}* . **Diabetes** 47:1699-1706

Novelli M, De Tata V, Bombara M, Lorenzini A, Masini M, Pollera M, Bergamini E, Masiello P. (1999) *Insufficient adaptative capability of pancreatic endocrine function in dexamethasone-treated ageing rats*. **J. Endocrinol.**, 162:425-432

Ogawa A, Johnson JH, Ohneda M, McAllister CT, Inman L, Alam T, Unger RH (1992) *Roles of insulin resistance and β -cell dysfunction in dexamethasone-induced diabetes*. **The Journal of Clinical Investigation** 90:497-504

Ohneda M, Jonson JH, Inman LR, Unger RH. (1993) *Glut-2 function in glucosa-unresponsive β -cells of dexamethasone-induced diabetes in rats*. **J Clin Invest.** 92:1950-1956

Oomura Y. (1983) *Glucose as a regulator of neuronal activity*. **Adv. Metab. Dis.** 10:31-65

Ostenson CG, Grill V. (1987) *Evidence that hyperglycemia increases muscarinic binding in pancreatic islets of the rat*. **Endocrinology.** 121:1705-1710

Pagano G, Cavallo PP, Cassader M, Bruno A, Ozzelo A, Masciola P, Dall'Omo AM, Imbimbo B. (1983) *An in vivo and in vitro study of the mechanism of prednisone-induced insulin resistance in healthy subjects*. **J. Clin. Invest.** 72:1814-1820

Persaud SJ, Jones PM, Howell SJ. (1991) *Activation of protein kinase C is essential for sustained insulin secretion in response to cholinergic stimulation*. **Biochim Biophys Acta.** 1091:120-122

Rafacho A, Ribeiro DL, Boschero AC, Taboga SR, Bosqueiro JR (2008) *Increased pancreatic islet mass is accompanied by activation of the IRS-2/AKT pathway and augmented cyclin D2 protein levels in insulin resistant rats*. **Int J Exp Pathol.** 89(4):264-275

Rafacho A, Ribeiro DL, Boschero AC, Taboga SR, Bosqueiro JR (2008) *Increased pancreatic islet mass is accompanied by activation of the IRS-2/AKT pathway and augmented cyclin D2 protein levels in insulin resistant rats.* **Int J Exp Pathol** 89(4):264-75

Rafacho A, Roma LP, Taboga SR, Boschero AC, Bosqueiro JR (2007) *Dexamethasone-induced insulin resistance is associated with increased connexins 36 mRNA and protein expression in pancreatic rat islets.* **Can. J. Physiol. Pharmacol.** 53:881- 885

Renuka RT, Robinson R, Paulose CS (2006) *Increased Insulin Secretion by Muscarinic M₁ and M₃ Receptor Function from Rat Pancreatic Islet in vitro.* **Neurochem Res.** 31:313-320

Rhee SG, Choi KD (1992) *Regulation of inositol phospholipid-specific phospholipase C isozymes.* **J.Biol.Chem.** 267:12393-12396

Rhodes CJ (2005) *Type 2 diabetes-a matter of beta-cell life and death?* **Science** 307:380–384.

Ronhner-Jeanrenaud F, Hochstrasser AC, Jeanrenaud B. (1983) *Hyperinsulinemia of preobese and obese fa/fa rats is partly vagus nerve mediated.* **Am J Physiol.** 244: 317-322

Rosetti L, Shulman GI, Zawulich W, DeFronzo RA. (1987) *Effect of chronic hyperglycaemia on in vivo insulin secretion in partially pancreatectomized rats.* **J. Clin. Invest.** 80:1037-1044

Ruzzin J, Wagman AS, Jensen J. (2005) *Glucocorticoid-induced insulin resistance in skeletal muscles: defects in insulin signaling and the effects of a selective glycogen synthase kinase-3 inhibitor.* **Diabetologia** 48:2119-2130

Saad MJ, Folli F, Kahn JA, Kahn CR. (1993) *Modulation of insulin receptor, insulin receptor substrate-1, and phosphatidylinositol 3-kinase in liver and muscle of dexamethasone-treated rats.* **J. Clin. Invest.** 92:2065-2072

Samy M, Halim A, Guenifi A, He B, Yang B, Mustafa M, Höjeberg B, Hillert J, Bakhiet M, Efendić S. (1998) *Mutations in the Promoter of Adenyl Cyclase (AC)-III Gene, Overexpression of AC-III mRNA, and Enhanced cAMP Generation in Islets From the Spontaneously Diabetic GK Rat Model of Type 2 Diabetes.* **Diabetes** 47:498- 504

Schaaf MJ, Cidlowski CR. (2002) *Molecular Mechanism of glucocorticoid action and resistance.* **J Steroid Biochem Mol. Biol.** 83:37-48

Scott AM, Atwater I, Rojas E. (1981). *A method for the simultaneous measurement of insulin release and B cell membrane potential in single mouse islets of Langerhans* **Diabetologia** 21:470-475

Seino S, Chen L, Seino M, Blondel O, Takeda J, Johnson JH, Bell GI. (1992) *Cloning of the $\alpha 1$ subunit of a voltage-dependent calcium channel expressed in pancreatic β cells.* **Proc Natl Acad Sci USA** 89:584–588

Sesti G, Federici M, Hribal ML, Lauro D, Sbraccia P, Lauro R. (2001) *Defects of the insulin receptor substrate (IRS) system in human metabolic disorders.* **FASEB J.** 15:2099-2111

Severino C, Brizzi P, Solinas A, Secchi G, Maioli M, Tonolo G. (2002) *Low-dose dexamethasone in the rat. A model to study insulin resistance.* **Am. J. Physiol.** 283:367-373

Sharp GW. (1996) *Mechanisms of inhibition of insulin release.* **Am J Physiol.** 271:1781-1799

Sharp GW. (1979) *The adenylate cyclase-cyclic AMP system in islets of Langerhans and its role in the control of insulin release.* **Diabetologia** 16:287–296

Skoglund G, Lundquist I, Åhrén B. (1988) *Selective $\alpha 2a$ - adrenoceptor activation by clonidine: effects on $^{45}\text{Ca}^{2+}$ efflux and insulin secretion from isolated rat islets.* **Acta Physiol Scand.** 132:289-296

Shepherd PR, Withers DJ, Siddle K. (1998) *Phosphoinositide 3-kinase: the key switch mechanism in insulin signalling*. **Biochem J.** 333:471–490.

Straub SG, Sharp GW. (1996) *Awortmannin-sensitive signal transduction pathway is involved in the stimulation of insulin release by vasoactive intestinal polypeptide and pituitary adenylate cyclase-activating polypeptide*. **J Biol Chem.** 271:1660-1668

Spellman CW. (2007) *Islet cell dysfunction in progression to diabetes mellitus*. **J Am Osteopath Assoc** 107:1-5

Sundler F, Bottcher G. (1991) *Islet innervation with special reference to neuropeptides*. **The Endocrine Pancreas** 29-52

Swenne I, Crace CJ, Milner RD. (1987) *Persistent impairment of insulin secretory response to glucose in adult rats after limited period of protein-calorie malnutrition early in life*. **Diabetes** 36:454-458

Toriumi C, Imai K. (2003) *Altered expresion of insulins I and II and their mRNAs in the islets of Langerhans in dexamethasone-induced diabetic rats*. **Biomed Chromat.**17:26-32

Valverde I, Vandermeers A, Anjaneyulu R, Malaisse WJ. (1979) *Calmodulin activation of adenylate cyclase in pancreatic islets*. **Science** 206:225–227

Verchere CB, Kwok YN, Brown JC. (1991) *Modulation of acetylcholine-stimulated insulin release by glucose and gastric inhibitory polypeptide*. **Phamacology** 12:273-282

Wahl MA, Landsbeck EA, Ammon HPT, Verspohl EJ. (1992) *Gastrin-releasing peptide: binding and functional studies in mouse pancreatic islets*. **Pancreas** 7:345-351

Weinstein SP, Wilson CM, Pritsker A, Cushman SW. (1998) *Dexamethasone inhibits insulin-stimulated recruitment of GLUT4 to the cell surface in rat skeletal muscle*. **Metabolism** 47:3-6.

Westfall TC. (1984) *Evidence that noradrenergic transmitter release is regulated by presynaptic receptors.* **Fed Proc.** 43:1352-1357

Wild S, Roglic G, Green A, Sicree R, King H. (2004) *Global prevalence of diabetes: estimates for the year 2000 and projections for 2030.* **Diabetes Care** 27:1047-1053.

Yamazaki H, Philbrick W, Zawulich KC, Zawulich WS. (2006) *Acute and chronic effects of glucose and carbachol on insulin secretin and phospholipase C activation. Studies with diazoxide and atropine.* **Am J Physiol Endocrinol metabolism** 290:26-33

Zawulich WS, Tesz GJ, Yamazaki H, Zawulich KC, Philbrick W. (2005) *Dexamethasone suppresses phospholipase C activation and insulin secretion from isolated rat islet.* **Metabolism** 35:35-42

Zawulich WS, Zawulich kC, Ramussen H. (1989) *Cholinergic agonist prime the beta cell to glucose stimulation.* **Endocrinology** 125:2400-2406

