Instituto de Biociências

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de Botucatu

PG-BGA

# AVALIAÇÃO DA PARTICIPAÇÃO DOS ÁCIDOS GRAXOS NAS ADAPTAÇÕES DAS ILHOTAS PANCREÁTICAS À RESISTÊNCIA PERIFÉRICA À INSULINA PELO TRATAMENTO COM DEXAMETASONA

**MAIARA DESTRO**

Dissertação apresentada ao Instituto de Biociências, Campus de Botucatu, UNESP, para obtenção do título de Mestre no Programa de Pós-Graduação em Biologia Geral e Aplicada, Área de concentração em Biologia Celular Estrutural e Funcional.

*Orientador: Prof. Dr. José Roberto Bosqueiro*

**BOTUCATU – SP**
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*Banca Examinadora*

Prof. Dr. José Roberto Bosqueiro (Orientador)

Profa. Dra. Margarida Juri Saeki

Profa. Dra. Débora Cristina Damasceno

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*Às pessoas que mais me amam neste mundo:*

*Meus pais, José Valentim Destro e*

*Ester Fátima de Aguiar Destro.*

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## *Epígrafe*

*"Confia no senhor de todo seu coração e não se estribes no teu próprio entendimento. Reconhece-o em todos os teus caminhos, e ele endireitará as tuas veredas."*

*(Provérbios 3:5-6)*

*Sumário*

## *Lista de abreviaturas*

**AC -** adenilato ciclase

**AGL -** ácidos graxos livres

**AMPc -** adenosina monofosfato cíclico

**ATP -** adenosina trifosfato

**$Ca^{2+}$ -** íon cálcio

**CPT-1 -** carnitina-palmitoiltransferase-1

**DAG -** diacilglicerol

**DM -** *diabetes mellitus*

**GLUT-2 -** proteína transportadora de glicose 2

**GPR40 -** receptor acoplado à proteína G

**HDL -** lipoproteína de alta densidade

**IP3 -** inositol 1,4,5-trifosfato

**K-ATP -** canais de potássio sensíveis a ATP

**LC-CoA -** acil-CoA de cadeia longa

**NAD(H)P -** adenina dinucleotídeo fosfato

**PKA -** proteína quinase A

**PKC -** proteína quinase C

**PLC -** fosfolipase C

**PPAR -** receptores ativados por proliferador de peroxissoma

**RI -** resistência à insulina

**SIEG -** secreção de insulina estimulada por glicose

**VLDL -** lipoproteína de densidade muito baixa

*Resumo*

O aumento da secreção de insulina estimulada por glicose é um mecanismo adaptativo observado nas ilhotas pancreáticas de animais resistentes à insulina. Estudos relatam que os ácidos graxos livres estimulam a secreção de insulina através da ativação do GPR40. Diante destes fatos, investigamos a secreção de insulina, a expressão de proteínas da via do GPR40 nas células ß e a participação dos lipídios na resistência à insulina induzida por dexametasona, através do tratamento com o redutor de lipídios bezafibrato. Os grupos receberam gavagem uma vez ao dia durante 28 dias: Controle (CTL) e DEXA com goma arábica 5% (1 ml/kg, peso corpóreo); BEZA e BEZA-DEXA com bezafibrato (300 mg/kg, p.c.). Nos últimos 5 dias de tratamento os grupos receberam injeções intraperitoniais: CTL e BEZA de solução salina (1 ml/kg, p.c.); DEXA e BEZA-DEXA de dexametasona (Decadron® 1,0 mg/kg, p.c). A secreção de insulina estimulada por glicose aumentou nos grupos BEZA e DEXA. BEZA-DEXA exibiu diminuição dos níveis de ácidos graxos livres, triglicérides e de insulina, mas não houve elevação dos níveis de glicose no sangue. Além disso, houve melhora na resistência à insulina e restauração do padrão de secreção de insulina, em comparação ao grupo DEXA. Nas ilhotas dos animais BEZA-DEXA a expressão das proteínas GPR40, PLCß1 e PKCζ foi significativamente maior em relação aos valores obtidos em DEXA. Esta via permaneceu inalterada nas ilhotas de DEXA e BEZA. Em conclusão, o tratamento com bezafibrato melhorou a função das células ß e impediu a indução de resistência à insulina pelo tratamento com dexametasona, mas os mecanismos não são conhecidos. O aumento na secreção de insulina em DEXA aparentemente não está relacionado com a ativação do GPR40. Contrariando a literatura, apesar da redução na secreção de insulina, as ilhotas dos animais BEZA-DEXA apresentaram ativação da via do GPR40.

**Palavras-chave:** Ácidos graxos, glicocorticoides, secreção de insulina, ilhotas pancreáticas, bezafibrato.

## *Abstract*

Increased glucose-stimulated insulin secretion is an adaptive mechanism exhibited by pancreatic islets from insulin resistant animal. Studies report that the free fatty acids stimulate the insulin secretion via GPR40. As such, we investigate the expression of GPR40 in ß-cells and the involvement of lipids in dexamethasone-induced IR, by lipid-lowering therapy with bezafibrate. Groups received once daily gavage for 28 days: Control (CTL) and DEXA with gum Arabic 5% (1.0 mg/kg, body weight); BEZA and BEZA-DEXA with bezafibrate (300 mg/kg, b.w.). In the last 5 days of the treatment groups received intraperitoneal injections: CTL and BEZA of saline (1.0 mg/kg, b.w.); DEXA and BEZA-DEXA of dexamethasone (Decadron® 1.0 mg/kg, b.w.). The glucose-stimulated insulin secretion increased in the DEXA and BEZA groups. BEZA-DEXA shows decrease in fatty acids, triglycerides and insulin levels, but not raised blood glucose levels. In addition, there was improved in insulin resistance and restoration the insulin secretory pattern, when compared to DEXA group. In BEZA-DEXA islets, GPR40, PLCß1 and PKCζ protein content was significantly higher than DEXA. This pathway remained unchanged in DEXA and BEZA islets. In conclusion, bezafibrate treatment improved ß-cell function and prevented dexamethasone-induced IR, but the mechanisms are not known. Augmented insulin secretion in DEXA appears to be unrelated to the activation of the GPR40. Contrary to the literature, despite the reduction in insulin secretion, BEZA-DEXA islets showed activation of the GPR40 pathway.

**Keywords:** Fatty acids, glucocorticoids, insulin secretion, pancreatic islet, bezafibrate.

## *Introdução*

### 1. Secreção de insulina

A insulina é o hormônio polipeptídio anabólico mais conhecido e essencial para a manutenção da homeostase da glicose, além de promover crescimento e diferenciação celular (Carvalheira et al., 2002). Esse hormônio é secretado pelas células β das ilhotas pancreáticas em resposta ao aumento dos níveis circulantes de glicose, aminoácidos e ácidos graxos (Prentki et al., 1997) após as refeições. A distribuição eficiente desses nutrientes nos tecidos periféricos (fígado, músculo e tecido adiposo) é coordenada pela ação antagônica da insulina e do glucagon, um hormônio secretado pelas células α das ilhotas pancreáticas (Jiang & Zhang, 2003). A insulina aumenta a captação periférica de glicose, reduz a produção hepática da mesma via diminuição da gliconeogênese e glicogenólise, além de estimular a lipogênese e a síntese proteica (Carvalheira et al., 2002). Em baixas concentrações de glicose, o glucagon disponibiliza os substratos energéticos via glicogenólise, gliconeogênese e lipólise sem, contudo, interferir no metabolismo muscular (Jiang & Zhang, 2003).

A glicose é o principal agente estimulador da secreção de insulina (figura 1), que reponde rapidamente ao aumento das suas concentrações (Carvalheira et al., 2002). Esse açúcar é transportado para interior da célula β pela proteína transportadora de glicose 2 (GLUT-2). Sua metabolização eleva os níveis de adenosina trifosfato (ATP) que age fechando os canais de potássio sensíveis a ATP (K-ATP). A diminuição do efluxo de potássio tem como consequência a despolarização da membrana e abertura dos canais de cálcio ($Ca^{2+}$) sensíveis à voltagem. O influxo de $Ca^{2+}$ e o aumento da concentração de $Ca^{2+}$ intracelular ativa a maquinaria de exocitose, resultando na secreção dos grânulos de insulina (Matschinsky, 1996).

Um efeito secundário da estimulação das células β pela glicose é a ativação da fosfolipase C (PLC), que hidrolisa os fosfolipídios da membrana plasmática gerando diacilglicerol (DAG) e inositol 1, 4, 5-trifosfato (IP3) (Zawalich & Zawalich, 1996). O IP3 promove a ativação dos canais de $Ca^{2+}$ localizados na membrana do reticulo endoplasmático, e o DAG atua sobre os canais de $Ca^{2+}$ sensíveis à voltagem da membrana. Além disso, DAG também ativa a proteína quinase C (PKC), a responsável por estimular a exocitose dos grânulos secretórios de insulina, e ativa a enzima adenilato ciclase (AC). A ativação da AC aumenta o conteúdo intracelular de adenosina monofosfato cíclico (AMPc), que por sua vez, ativa a proteína quinase A (PKA). A PKA pode estimular a secreção de insulina pela

fosforilação do canal de $Ca^{2+}$ sensível à voltagem, permitindo a entrada do íon na célula (Flatt, 1996).

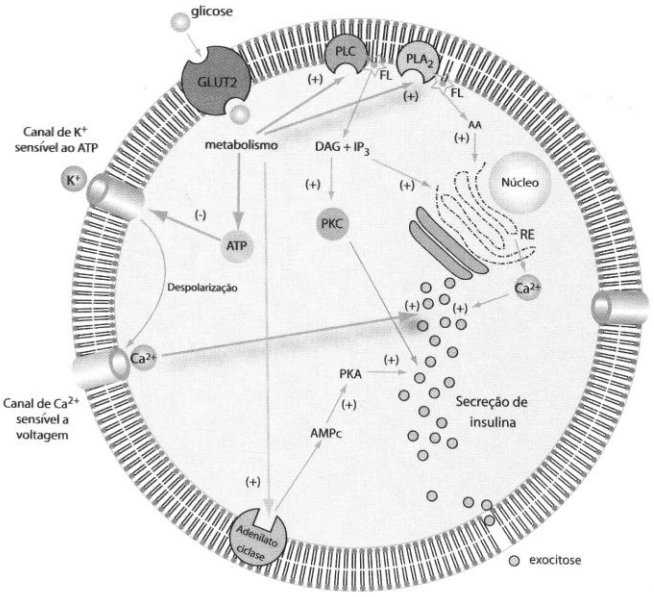


**Figura 1.** Principais eventos envolvidos na secreção de insulina estimulada por glicose. GLUT-2 = transportador de glicose do tipo 2; ATP = adenosina trifosfato; PLC = fosfolipase C; PLA2 = fosfolipase A2; FL = fosfolipídios; AA = ácido araquidônico, RE = retículo endoplasmático, PKA = proteína quinase A; PKC = proteína quinase C; DAG = diacilglicerol; IP3 = inositol trifosfato; AMPc = adenosina monofosfato cíclico; (+) estimula; (-) inibe. Fonte: Curi *et al.* (2002).

## 2. Efeitos dos ácidos graxos sobre a secreção de insulina

Os ácidos graxos são necessários para o funcionamento normal das células β pancreáticas (Prentki et al., 2002; McGarry et al., 2003; Nolan et al., 2006a). Eles potencializam a secreção de insulina estimulada por glicose (SIEG) de maneira tempo-dependente e de acordo com o tamanho da cadeia e o grau de insaturação do ácido graxo (Stein et al., 1996). A exposição aguda a ácidos graxos livres (AGL) resulta no aumento da secreção de insulina estimulada por glicose. Porém, em exposição crônica aos AGL e particularmente em associação com glicose elevada ocorre redução da secreção de insulina (El-Assad et al., 2003), apoiando a hipótese de glicolipotoxicidade citado por Prentki e colaboradores (2002).

Nas células β pancreáticas os AGL do citoplasma são convertidos a acil-CoA pela enzima acil-CoA sintetase. Em condições basais, a molécula de acil-CoA de cadeia longa (LC-CoA) é transportada para a mitocôndria via carnitina-transferase-1, onde é oxidada. Contudo, na presença de concentrações elevadas de glicose (figura 2), o citrato, um produto da metabolização da glicose no ciclo do ácido cítrico, desloca-se da mitocôndria para o

citoplasma onde é transformado em malonil-CoA pelas enzimas citratase e acetil-CoA carboxilase. Malonil-CoA inibe principalmente a carnitina-palmitoiltransferase-1 (CPT-1), o que permite o acúmulo de LC-CoA no citoplasma. Moléculas de LC-CoA posteriormente podem ser esterificadas com glicerol-3-fosfato para formar lipídios complexos como triglicerídeos, diacilglicerol, fosfolípidios e o ácido fosfatídico (Prentki & Corkey, 1996; McGarry & Brown, 1997). Os lipídios complexos formados fornecem fatores de sinalização que potencializam a secreção de insulina (Ramanadham et al., 1999). Os produtos de esterificação nas células β pancreáticas, tais como os triglicerídeos, podem ainda sofrer lipólise fornecendo AGL pela ação da lipase hormônio sensível, um papel também importante no estímulo na secreção de insulina (Roduit et al., 2001).

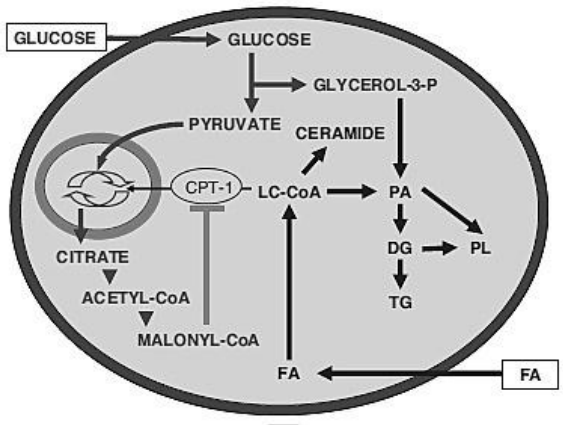


**Figura 2.** Efeitos da glicose no metabolismo dos lipídios na célula ß. FA = ácido graxo; CPT-1 = carnitina-palmitoiltransferase-1; LC-CoA = acil-Coa de cadeia longa; PA = ácido fosfatídico; DG = diacilglicerol; TG = triglicerídeos; PL = fosfolipídios. Fonte: Poitout *et al.* (2010).

O aumento do *pool* citossólico de LC-CoA, diacilglicerol e ácido fosfatídico aumentam diretamente a exocitose dos grânulos de insulina por estimularem a liberação de $Ca^{2+}$ do retículo endoplasmático (Yaney et al., 2000), modificarem o estado de acilação de proteínas chaves que regulam a atividade dos canais iônicos e da exocitose (Bouvieral et al., 1995), e também por modularem diretamente a atividade de enzimas como a PKC. Estudos demonstraram que a expressão PKC ζ é regulada pelo LC-CoA (Yaney et al., 2000). Em adição, há evidências que esta isoforma regula a proliferação das células β (Vasavada et al., 2007).

Além dos sinais lipídicos formados no metabolismo intracelular, estudos realizados por Itoh *et al.* (2003) levantaram a hipótese de que a ativação do receptor de AGL altamente expresso nas células β, o receptor acoplado à proteína G (GPR40), também potencializa a

secreção de insulina estimulada por glicose (SIEG) (figura 3). Em ilhotas de camundongos *knockout* para GPR40 é observada uma redução de 50% da SIEG (Latour et al., 2007).

Ainda é pouco esclarecido na literatura o mecanismo de ativação do GRP40. Contudo, estudos demonstraram que a ligação do ácido graxo ao receptor estimula a secreção de insulina pela ativação da PLC, que gera DAG e IP3, os responsáveis por induzir a liberação de $Ca^{2+}$ e ativar isoformas de PKC, respectivamente. A ativação do GPR40 também age na proliferação celular (Gromada, 2008) e na regulação dos canais K-ATP (Feng et al., 2006).

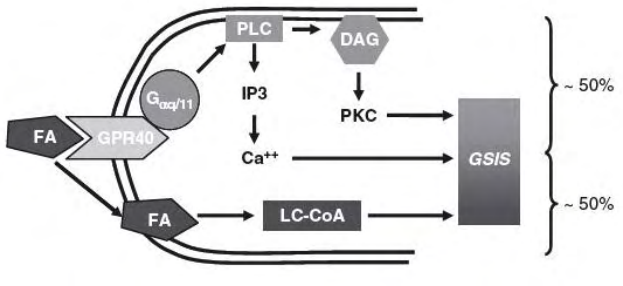


**Figura 3.** Metabolismo intracelular dos ácidos graxos e os sinais mediados pelo GPR40 que potencializam a secreção de insulina na célula ß. FA = ácido graxo; GPR40 = receptor acoplado à proteína G 40; PLC = fosfolipase C; IP3 = inosito trifosfato; DAG = diacilglicerol; PKC = proteína quinase C; GSIS = secreção de insulina estimulada por glicose; LC-CoA= acil-Coa de cadeia longa. Fonte: Kebede *et al.* (2009).

## 3. Resistência à insulina

A resistência à insulina (RI) caracteriza-se pela redução da capacidade dos tecidos sensíveis à insulina de regularem a homeostase da glicose em níveis normais do hormônio. As principais características da RI são o aumento da lipólise no tecido adiposo, aumento da gliconeogênese no fígado e diminuição da captação de glicose pelo músculo. As células β, frente às concentrações crescentes de glicose impostas pela RI, desenvolvem mecanismos compensatórios, dentre os quais estão a hiperinsulinemia, para manter os níveis normais de glicemia no sangue (Cefalu, 2001) e, dependendo do grau de RI, ocorre em conjunto com hipertrofia das ilhotas pancreáticas e aumento da proliferação das células β, fato que também auxilia na homeostase da glicose (Rafacho et al., 2008a; Rafacho et al., 2009). Após estas adaptações, ocorre nas células β pancreáticas uma progressiva deterioração da sua função associada à perda de massa celular devido a apoptose, o que as tornam incapazes de secretar quantidades adequadas do hormônio. Instala-se assim, o quadro de *diabetes mellitus* tipo 2 (DM tipo 2) (Cefalu, 2001) (figura 4).

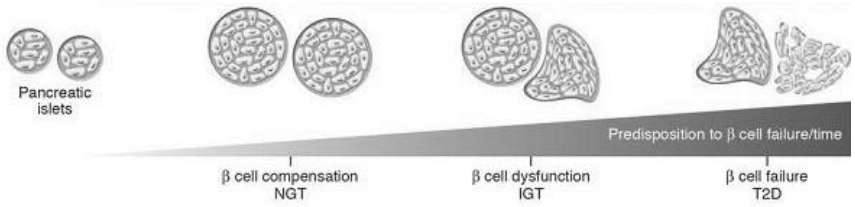


**Figura 4.** Disfunção da célula ß e o desenvolvimento do *diabetes mellitus* tipo 2. NGT = não tolerante à glicose; IGT = intolerante à glicose; T2D = *diabetes mellitus* tipo 2. Fonte: Prentki & Nolan (2006).

O *diabetes mellitus* é uma doença metabólica com alterações no metabolismo de carboidratos, lipídeos e proteínas, decorrentes de defeitos na secreção e ação de insulina (AMERICAN DIABETES ASSOCIATION, 2006). Nas últimas décadas é visto um aumento dramático na incidência e prevalência do diabetes no mundo. A Federação International de Diabetes estimou em 2010 cerca de 285 milhões de pessoas diabéticas em todo o mundo, representando 6,6% da população. Este número deve chegar a 438 milhões em 2030, ou 7,3% dos adultos do mundo (INTERNACIONAL DIABETES FEDERATION, 2009). Entre os tipos de diabetes, o DM tipo 2 corresponde a mais de 90% dos casos e estabelece-se pela combinação de predisposição genética associada ao sedentarismo e à obesidade (Reaven, 1988). O DM tipo 2 é caracterizado por hiperglicemia devido à resistência à insulina e redução na função da célula β (Gross et al., 2002). Na maioria das pessoas obesas também é observada a resistência à insulina. A dislipidemia é uma característica comum entre os indivíduos resistentes à insulina, diabéticos do tipo 2 e obesos (Yaney & Corney, 2003). Esta disfunção é caracterizada por uma alteração no metabolismo das lipoproteínas gerando um aumento nos níveis plasmáticos de triglicérides e ácidos graxos livres, e redução nos níveis de colesterol HDL (lipoproteína de alta densidade) (Henkin & Oberman, 1992).

O DM tipo 2 pode ser controlado inicialmente por uma dieta saudável, perda de peso e aumento da atividade física. A maioria das pessoas com DM tipo 2 exigirá tratamento farmacológico (Gillies et al., 2008). O controle da glicemia melhora com medicamentos hipoglicemiantes, mas raramente normaliza o metabolismo alterado das lipoproteínas, e alguns casos a dislipidemia geralmente persiste necessitando também de agentes hipolipedimiantes (Fredrick, 2010).

Estudos têm descrito que os AGL contribuem para a RI (Bollheimer et al., 1998; Prentki et al., 2002; McGarry et al., 2003; Nolan et al., 2006b; Lewis et al., 2002). O fato que os ácidos graxos estimulam a secreção de insulina *in vivo* e *in vitro* corrobora com a presença da hiperinsulinemia e elevados níveis de lipídios circulantes no plasma nos indivíduos resistentes à insulina (Yaney & Corney, 2003). A redução da taxa de lipidios melhora o quadro de RI e função das células ß (Flory et al., 2009). No entanto, não está totalmente

esclarecido na RI qual o mecanismo celular que os ácidos graxos livres alteram a secreção de insulina.

Diversos trabalhos concordam que a hiperinsulinemia é a responsável por induzir a resistência à insulina. De acordo com Steneberg *et al.* (2005), a resistência à insulina induzida por dieta com alto teor de gordura ocorre devido à hiperinsulinemia provocada pela ativação do GPR40 (figura 5a). A ausência de GPR40 em camundongos protege da obesidade induzida por hiperinsulinemia, esteatose hepática, hipertrigliceridemia, hiperglicemia e intolerância à glicose. Esta hipótese afirma que a deleção do GPR40 previne a RI e seria uma estratégia terapêutica (Aston-Mourney et al., 2008).

Em contraste, diversos trabalhos contradizem essa observação. Dentre eles, Kebede *et al.* (2009) propôs que o aumento da secreção de insulina pela ativação do GPR40 contribui para os mecanismos compensatórios nas células β observados na resistência à insulina (figura 5b). Essa observação é apoiada por estudos em humanos que associaram alterações da secreção de insulina com a perda da função do gene GPR40 por mutações (Vettor et al., 2008). Entretanto, as discrepâncias entre os estudos demonstram a necessidade de investigações adicionais sobre o papel fisiológico e mecanismos de ação desses receptores acoplados à protéina G.

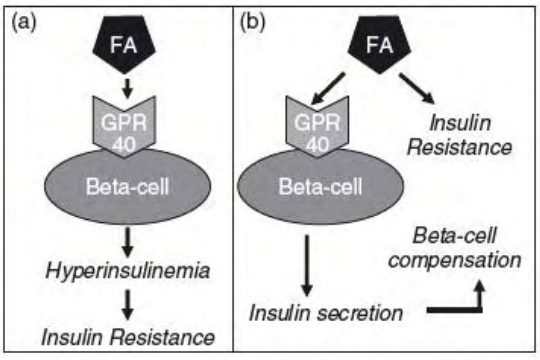


**Figura 5.** Duas hipóteses de indução a resistência à insulina pelos lipídios e compensação das células ß. (a) O aumento da secreção de insulina pela ativação do GPR40 causa a resistência à insulina. (b) A secreção de insulina ativada pelo GPR40 contribui para os mecanismos compensatórios nas células ß, observados na resistência à insulina. FA= ácido graxo, GPR40 = receptor acoplado à proteína G 40. Fonte: Kebede *et al.* (2009).

## 4. Glicocorticoides

Os glicocorticoides são hormônios esteroides produzidos no córtex das glândulas adrenais sob o controle do eixo hipotálamo-pituitária-adrenal (Andrews & Walker, 1999). Na

prática clínica eles são amplamente utilizados como agentes antiinflamatórios e imunossupressores (Saklatvala, 2002). Em excesso, os glicocorticoides induzem resistência à insulina (Stojanovska et al., 1990; Korach-Andre et al., 2005; Rafacho et al., 2008b), como evidenciado na síndrome de Cushing (Saklatvala, 2002), por aumentar a produção hepática de glicose pela gliconeogênese e diminuir sua captação pelos tecidos periféricos (Delaunay et al., 1997). Além disso, dependendo da dose e do tempo de tratamento, os glicocorticoides podem causar DM tipo 2 (Hoogwerf & Danese 1999).

Em animais induzidos à resistência periférica à insulina com o glicocorticoide sintético dexametasona (1mg/kg p.c por 5 dias consecutivos) são observadas alterações funcionais e morfológicas que ocorrem nas ilhotas pancreáticas como mecanismos compensatórios à RI (Rafacho et al., 2008a; Rafacho et al., 2009). Rafacho e colaboradores (2010) relataram que o aumento da função mitocondrial, sinalização de $Ca^{2+}$, atividades das proteínas PLC/PKC, bem como alterações na exocitose, podem ter um papel importante no aumento da SIEG observado nas ilhotas de animais tratados com dexametasona. Observou-se neste trabalho que as ilhotas aumentaram os níveis de $Ca^{2+}$ intracelular em resposta à glicose elevada. No entanto, quando estimuladas com KCl e tolbutamida as ilhotas não apresentaram alterações na atividade dos canais de $Ca^{2+}$ sensíveis à voltagem e canais K-ATP, respectivamente. Em adição, ilhotas expostas ao carbacol, um ativador do receptor muscarínico, potencializou a secreção de insulina, a atividade da PLC e o aumento de $Ca^{2+}$ intracelular. Ao analisar a função mitocondrial de ilhotas tratadas com dexametasona e incubadas em glicose elevada observou-se um aumento na geração de NAD(P)H. Porém, esta alteração mitoncondrial não tem relação com as enzimas glicoquinase e piruvato quinase, importantes no metabolismo da glicose.

Os glicocorticoides apresentam um papel modulador sobre os lipídios circulantes. Eles aumentam os níveis plasmáticos de triglicérides por inibição da atividade da lipoproteína lipase (Franco-colin et al., 2000) e aumentam os níveis de AGL por ativação da lipase hormônio sensível (Slavin et al., 1994). No modelo de indução a RI com dexametasona é verificado uma marcante elevação nos níveis de AGL e triglicérides. Esta observação levou à hipótese dos lipídios modularem a compensação das ilhotas resistentes à insulina, também citada em outros trabalhos (Prentki et al., 2002; El-Assad et al., 2003; Novelli et al., 2008). Contudo, não há neste modelo um estudo da importância dos ácidos graxos sobre alterações funcionais nas ilhotas pancreáticas.

## 5. Receptor ativado por proliferador de peroxissoma

Os receptores nucleares ativados por proliferador de peroxissoma (PPARs) têm sido implicados em diversos processos, incluindo metabolismo de lipídios e carboidratos, gasto energético, processos imunes e inflamatórios, homeostase vascular, remodelação tecidual e diferenciação e proliferação celular em tecidos normais e neoplásicos (Scatena et al., 2008). Em vertebrados e mamíferos foram identificados três isoformas: PPARα, PPARβ/δ e PPARγ (NUCLEAR RECEPTORS NOMENCLATURE COMMITTEE, 1999) com distribuição tecidual e funções distintas (Kliewer, et al. 1994). Nas células β são expressas todas as isoformas de PPAR (Zhou et al., 1998).

O PPARα é responsavel por controlar um conjunto de genes que regulam o metabolismo dos ácidos graxos e lipoproteínas nas mitocôndrias, peroxissomos e retículo endoplasmático durante o jejum, induzindo a proliferação peroxissomal, oxidação dos ácidos graxos e a produção de corpos cetônicos (Yoon, 2009). Esta isoforma estimula o influxo de ácidos graxos na mitocôndria via CPT-1 reduzindo assim a sua disponibilidade para síntese de triglicerídeos. O PPARα também reduz os níveis de triglicérides por aumentar o catabolismo das partículas de VLDL (lipoproteína de densidade muito baixa) e da expressão da enzima lípase lipoprotéica (Schoonjans et al., 1996) através da redução da expressão da Apoproteína C-III (Staels et al., 1995) e aumento da expressão da apoproteína A-V (Prieur et al., 2003). Além disso, a ativação de PPARα aumenta os níveis de colesterol HDL por aumentar a síntese da apoproteína A-I e A-II (Tenenbaum et al., 2005).

O PPARα é ativado por ligantes naturais como ácidos graxos, espécimes lipídicos oxidados, eicosanóides e prostaglandinas (Chawla et al., 2001) ou através de sintéticos como os fibratos e WY14643 (ácido pirinixico) (Yoon, 2009). Os fibratos são utilizados na prática clínica em pacientes com dislipidemia (Fruchart et al., 2001). Este fármaco foi introduzido na década de 1970 com o Clofibrato, seguido por gemfibrozil, bezafibrato, fenofibrato e ciprofibrato. O bezafibrato tem sido caracterizado como agonista tipo pan-PPAR, ativando o PPARα e com alta afinidade ao PPARδ e PPARγ (Krey et al., 1997). Estudos demonstraram em humanos (Tenenbaum et al., 2007; Flory et al., 2009) e em ratos (Jia et al., 2004; Nakano et al., 2007; Santos et al., 2009) que o tratamento com bezafibrato reduz o risco de desenvolver diabetes tipo 2 por melhorar a sensibilidade periférica à insulina e função das células β. A ativação por outros agonistas PPARα também normalizaram a hipersecreção de insulina em modelos de indução a resistência à insulina (Holness et al., 2003; Sudgen et al., 2003).

Em ilhotas pancreáticas no período de jejum ocorre aumento na expressão do PPARα e consequentemente na oxidação dos ácidos graxos (Sugden et al., 2002). Segundo Gremlich *et*

*al.* (2005), a oxidação dos ácidos graxos elimina moléculas lipídicas que estimulam a secreção de insulina, ou seja, no jejum a sinalização do PPAR é necessária para suprimir a SIEG.

Pesquisadores relataram em ilhotas incubadas com ativador PPARα e glicose, redução da secreção de insulina, bem como nas oscilações de $Ca^{2+}$ intracelular (Freeman et al., 2006). Contudo, dados da literatura demonstram que ilhotas incubadas com bezafibrato e glicose tiveram aumento da secreção de insulina e aumento na expressão do RNA mensageiro de PPAR-α, acil-CoA oxidase, CPT-1 e piruvato carboxilase, moléculas que poderiam contribuir no aumento da SIEG (Yoshikawa et al., 2001). Shimomura e colaboradores (2004) demonstraram que ativadores de PPARα podem induzir a secreção de insulina por inibição dos canais de K-ATP. Em complemento, Kotarsky *et al.* (2003) sugeriu que as tioglitazonas, uma classe de medicamentos antidiabéticos ativadores de PPAR γ, ativam o receptor GPR40, reconhecido por induzir a secreção de insulina nas células ß. No entanto, o mesmo não foi observado com o ativador de PPAR α Clofibrato.

## *Justificativa*

No modelo de indução a resistência periférica à insulina pelo tratamento com dexametasona por 5 dias (1 mg/Kg ip) observa-se que o pâncreas endócrino, para compensar a resistência à insulina, sofre alterações morfológicas e funcionais. A hiperinsulinemia e o aumento na secreção de insulina estimulada por glicose indicam a alteração funcional das ilhotas pancreáticas nesta condição. Adicionalmente, foi observado em diversos trabalhos que no tratamento com dexametasona ocorre elevação de ácidos graxos livres circulantes (Bollheimer et al., 1998; Barbera et al., 2001; Rafacho et al., 2008b) que contribuem no aumento da secreção de insulina, apoiando a hipótese que a elevação crônica dos ácidos graxos livres pode ser um mediador das mudanças induzidas pelo tratamento com dexametasona (Novelli et al., 2008). Uma das vias pela qual os ácidos graxos livres estimulam a secreção de insulina é a ativação do GPR40, o receptor de ácidos graxos presente nas células ß (Itoh et al., 2003).

Não está totalmente esclarecido na literatura o mecanismo celular pelo qual os ácidos graxos livres alteram a secreção de insulina na resistência à insulina. Desse modo, buscamos possível correlação com a via do GPR40 para investigar se a redução na taxa de lipídios pelo tratamento com bezafibrato altera as adaptações funcionais observadas nas células ß no modelo de RI induzida por dexametasona.

## *Objetivos*

### *Geral*

Avaliar no modelo de indução a resistência periférica à insulina com dexametasona a participação dos lipídios nas alterações funcionais nas células ß através do tratamento com o redutor de lipídios bezafibrato.

### *Específicos*

- Avaliar a resposta secretória de insulina estimulada por glicose pelas ilhotas pancreáticas.
- Avaliar a sensibilidade periférica à insulina.
- Avaliar a tolerância à glicose.
- Determinar a expressão das proteínas da via do GPR40.

## *Resultados*

Os resultados obtidos durante a realização deste trabalho estão apresentados a seguir sob a forma de um artigo submetido segundo normas da revista *Canadian Journal of Physiology and Pharmacology*.

**TITLE: Bezafibrate counteracts the effects of dexametasone treatment on insulin resistance and secretion**

**Maiara Destro[1], José Roberto Bosqueiro[2] ***

1- Institute of Biosciences, São Paulo State University-Unesp, Botucatu, São Paulo, Brazil.

2- Department of Physical Education, Faculty of Sciences, São Paulo State University-Unesp, Bauru, São Paulo, Brazil.

*Correspondence to:

José Roberto Bosqueiro, Department of Physical Education, Faculty of Sciences, São Paulo State University-Unesp, Bauru, São Paulo, Brazil.

Address: Av. Eng. Luiz Edmundo Carrijo Coube, 14-01, CEP: 17033-360 Bauru/SP-Brasil

Phone: 55-14-31036041

FAX: 55-14-31036041

E-mail: bosqueir@fc.unesp.br

**ABSTRACT**

Increased glucose-stimulated insulin secretion (GSIS) is an adaptive mechanism exhibited by pancreatic islets from insulin-resistant animal. Studies report that free fatty acids stimulate insulin secretion via GPR40. Here, we investigate the expression of GPR40 and the participation of lipids in the functional adaptation in ß-cells in the model of dexamethasone-induced IR, by lipid-lowering therapy with bezafibrate. Groups received daily gavage for 28 days: Control (CTL) and DEXA with gum Arabic; BEZA and BEZA-DEXA with bezafibrate (300 mg/kg b.w.). In the last 5 days of the treatment groups received i.p. injections: CTL and BEZA of saline; DEXA and BEZA-DEXA of dexamethasone (1.0 mg/kg b.w.). BEZA-DEXA showed decrease in fatty acids, triglycerides and insulin levels, but not raised glycaemia levels, and improved insulin resistance and decreased GSIS, compared to DEXA. In BEZA-DEXA islets, GPR40/PLCß1/PKCζ protein content was significantly higher than DEXA. This pathway remained unchanged in DEXA and BEZA islets. In conclusion, bezafibrate treatment improved ß-cell function and prevented dexamethasone-induced IR, but the mechanisms are not known. Augmented insulin secretion in DEXA appears to be unrelated to the activation of the GPR40. Contrary to the literature, despite the reduction in insulin secretion, BEZA-DEXA islets showed activation of the GPR40 pathway.

**Key-words:** fatty acids, glucocorticoids, insulin secretion, pancreatic islet, bezafibrate, GPR40.

## INTRODUCTION

Effects of glucocorticoid hormones in the treatment of inflammatory and autoimmune diseases are well known in clinical practice. Furthermore, depending on the dose and duration of treatment, glucocorticoids can induce insulin resistance (IR) and cause type 2 diabetes (Stojanovska et al., 1990; Korach-Andre et al., 2005; Rafacho et al., 2008a). This diabetogenic effect is due to the stimulated gluconeogenesis and decreased peripheral glucose uptake (Delaunay et al., 1997). Glucocorticoids are widely used in research to induce experimental IR (Tappy et al., 1994; Severino et al., 2002). Recent research demonstrated that daily injections of dexamethasone (1.0 mg/kg body weight, 5 days) induce IR in rodents (Rafacho et al., 2008a). It has been proposed in glucocorticoid treatment that hyperinsulinaemia is a compensatory mechanism of the endocrine pancreas to counterbalance the IR (Tappy et al., 1994, Karlsson et al., 2001 Rafacho et al., 2010). The hyperlipidaemia and hyperglycemia observed in dexamethasone-treated rats could contribute to the IR and hyperinsulinemia (Bollheimer et al., 1998; Barbera et al., 2001; Lewis et al., 2002). Moreover, the chronic exposure of free fatty acids (FFA) in combination with high blood glucose leads to β-cells failure, a phenomenon known as glucolipotoxicity (Prentki et al., 2002).

The control of insulin secretion is multifactorial and interconnected with nutrient like glucose, amino acid and FFA, as well as neurohumoral factors (Malaisse et al., 1973). The ability of FFAs to stimulate insulin secretion is well established (McGarry et al., 2003), but the cellular mechanisms are still unclear. However, studies demonstrated that fatty acids induce increase in insulin secretion by lipids involved in intracellular signaling (malonyl-CoA and long-chain acyl-CoA) (Prentki;

Corkey, 1996; Prentki et al., 2002) or by the activation of factors modulated by fatty acids such as the G-protein-coupled receptor GPR40 (Itoh et al., 2003; Gromada et al., 2008; Kebede et al., 2009) and peroxisome proliferator-activated receptors (PPAR) (Sugden et al., 2003; Gremlich et al.,2005).

The FFA receptor GPR40 is preferentially expressed in the pancreatic ß-cells (Itoh et al., 2003). The PPAR class of nuclear receptors is implicated in several processes, including metabolism of lipids and carbohydrates (Scatena et al., 2008). Three major isoforms of PPARs have been identified, PPARα, PPAR β/δ and PPARγ (Kliewer et al., 1994). All isoforms are expressed in pancreatic β-cells (Zhou et al., 1998). The PPARα is activated by fibrates, hypolipidemic drugs used in the treatment of dyslipidemia (Fruchart et al., 2001). Bezafibrate (BEZA) is a pan (α, β/δ and γ) PPAR activator that improves the lipid metabolism and in addition, reduces the risk of developing type 2 diabetes (Tenenbaum et al., 2007; Flory et al., 2009).

It is well known that the reduction in plasma lipids improves the islet function in IR. However, there are few studies on the mechanisms involved in this model of dexamethasone-induced IR. In the present study, we investigated the participation of FFA on the insulin secretion by pancreatic ß-cells of dexamethasone-induced insulin resistant rats, through lowering lipid levels by the treatment with bezafibrate, focusing on the GPR40 signaling pathway.

## MATERIALS AND METHODS

### Materials

Dexamethasone-phosphate (Decadron®) was from Aché (Campinas, SP, Brazil). Bezafibrate was from Pharma Nostra (Campinas, SP, Brazil). Gum Arabic

was from Sigma (St. Louis, MO). Human recombinant insulin (Biohulin N) was from Biobra´s (Montes Claros, MG, Brazil). The reagents used in the insulin secretion protocol and RIA were from Mallinckrodt Baker and from Sigma (St. Louis, MO). Sodium Thiopental (THIOPENTAX) was from Cristália (Itapira, SP, Brazil). SDS-PAGE and western blotting were performed using Bio-Rad Systems (Hercules, CA, USA), and all chemicals used were from Bio-Rad and from Sigma (St. Louis, MO). Enzymatic colorimetric assay for the quantification of free fatty acids (FFA), triglycerides (TG), total cholesterol (T-Chol), HDL cholesterol detection kits were from Human do Brasil (Itabira, SP, Brazil). The antibodies anti-G-protein-coupled receptor 40 (GPR40) (rabbit polyclonal), anti-protein kinase C zeta (PKCζ) (rabbit polyclonal), anti-phospholipase C ß1 (PLCß1) (rabbit polyclonal) and anti-α-tubulin antibody were from Santa Cruz Biotechnology (Santa Cruz, CA).

**Animals and treatment**

Experiments with animals were approved by the Institutional São Paulo State University (UNESP) Committee for Ethics in Animal Experimentation according to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH publication No. 85-23 revised 1996). The rats were obtained from the UNESP Animal Care Unit, Campus of Botucatu and kept at 22±2 Cº on a 12-hour light/dark cycle. The rats had access to food and water ad libitum.

The experiments were performed on 4 groups of male Wistar rats (3 months old). Once a day during 28 days, the experimental control groups (CTL) and DEXA received gavage of gum Arabic 5% (1 ml/kg b.w.) while BEZA and BEZA-DEXA groups received bezafibrate (300 mg/kg b.w., dissolved in gum Arabic 5%). In the last 5 days of the treatment, CTL and BEZA groups received daily intraperitoneal

injections of saline (1.0 ml/kg b.w.), and the DEXA and BEZA-DEXA groups received intraperitoneal injections of dexamethasone phosphate (Decadron®, 1.0 mg/kg b.w., dissolved in saline).

**Metabolic, Hormonal, and Biochemical measurements**

Body weight was measured daily and food intake was measured during dexamethasone treatment. On the day after the last treatment of each group, fasted (12-14 hours) and fed rats had the blood collected from the tail to measurement of blood glucose levels with a glucometer (One Touch; Johnson & Johnson). Immediately after, the rats were killed by exposure to carbon dioxide followed by decapitation. The serum obtained by centrifugation was used to measure all the following parameters: insulin levels were measured by radioimmunoassay (RIA), using a rabbit anti-rat insulin antibody and rat insulin as standard (Scott et al., 1981), TG, T-Chol, FFA and HDL cholesterol were determined by enzymatic colorimetric assay according to the manufacturer's directions. Hepatic glycogen was measured in fasted rats as described by Lo *et al.* (1970) with some modifications. The organs of fed animals (listed in table 1) were withdrawn and weighed.

**Intraperitoneal Glucose Tolerance Test (ipGTT)**

On the day after the last treatment of each group, fasted rats (12-14 hours) were anesthetized with sodium thiopental (60 mg/kg b.w). After checking the absence of reflexes, unchallenged samples (time 0) were obtained from rats' tails. Immediately after, 50% glucose (2 g/kg b.w.) was administered and blood samples were collected at 15, 30, 60 and 120 minutes from the tail tip for determination of glucose and insulin concentrations. For insulin determination, 75 µL of blood sample

was added to an Eppendorf tube containing 200 µL of saline (NaCl 0.9%), centrifuged at 15.000g and stored at ¯70 ºC for subsequent measurement of insulin content by RIA.

**Intraperitoneal Insulin Tolerance Test (ipITT)**

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

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

Islets were isolated by collagenase digestion of the pancreas as described (Lacy; Kostianovsky, 1967), with modifications. Insulin contents of islets were determined after extraction in acid-ethanol solution (12 mM HCl in 70% ethanol). The islets were sonicated for 15 seconds, centrifuged for 10 minutes at 3000g, and the supernatant was frozen for analysis of insulin content by RIA. For static incubation, groups of 5 islets were firstly incubated for 1 hour at 37 ºC in a Krebs-bicarbonate buffer solution of the following composition (in mM): 115 NaCl, 5 KCl, 2.56 CaCl, 1 $MgCl_2$, 24 $NaHCO_3$, 15 N-2 hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) and 5.6 glucose, supplemented with 0.5% of bovine serum albumin and equilibrated with a mixture of 95% oxygen and 5% carbon dioxide, pH 7.4. The medium was then replaced with fresh buffer containing the following solutions: 2.8 and 16.7 glucose. At

the end of the incubation, the samples were stored at ˉ20ºC for subsequent measurement of insulin content by RIA.

**Protein extration and imunnoblotting**

Pools of islets were homogenized in ice-cold cell lysis buffer (Cell Signaling) using a cell homogenizer (Fisher Scientific, Suwanee, GA, USA). Protein concentration from total cell lysate was determined by Bradford method according to the manufacturer (Bio-Rad, Hercules, CA, USA). Protein obtained from islets (100 µg) was used for each experiment. The sample was applied to a polyacrylamide gel and separated by SDS-PAGE in a BioRad miniature slab gel apparatus. The electrotransfer of proteins from the gel to nitrocellulose membrane was performed for 90 minutes at 120 V (constant) in a Bio-Rad miniature transfering apparatus (Mini-Protean) with 0.02% SDS and 20% methanol. After blocking for 2 h in Tris buffer salt tween (TBST) 5% dry skimmed milk, membranes containing islet lysates were washed in TBST (3-7 min) and incubated overnight with primary antibodies. After washing in TBST (3-10 min), membranes were incubated with the appropriate secondary antibody conjugated with HRP for 90 minutes in TBST 1% dried skimmed milk. Antibody binding was detected by enhanced SuperSignal® West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA), as described by the manufacturer. Blots were scanned (Epson expression 1600) and the densitometry of the protein bands was determined by the pixels intensity using Scion Image software (Scion Corporation, Frederick, MD, USA). The protein content values were corrected by expression of α-tubulin used as a control blot.

**Statistical Analysis**

Results are expressed as means ± SE of the indicated number of experiments. One-way ANOVA for unpaired groups, followed by Tukey's *post test* was used for multiple comparisons of parametric data. The significance level adopted was $P<0.05$.

## RESULTS

### Dexamethasone-induced IR decreases body weight and food intake

The DEXA and BEZA-DEXA groups in the last 5 days of treatment showed a reduction in body weight, but the significant reduction was observed from the third day of the treatment compared to other groups (Fig. 1). The animals in the DEXA group lost significantly more weight (14.69% ± 0.89) than the BEZA-DEXA group (10.85% ± 0.66), compared to CTL and BEZA groups ($n=8$, $P<0.05$). In addition, we observed that bezafibrate treatment does not influence the weight of the animals compared to CTL group.

The food intake was measured to try to explain the reduction in the body weight (Fig. 2). There was a significant reduction of food intake on the second day of the treatment with dexamethasone in the DEXA and BEZA-DEXA groups ($n=8$, $P<0.05$) compared to the BEZA group. However, on the last day of treatment the BEZA-DEXA group increased their food intake compared to DEXA animals ($n=8$, $P<0.05$). The bezafibrate treatment does not influence the food intake in relation to CTL group ($n=8$, $P<0.05$).

### Alterations of the overall organ mass induced by dexamethasone and bezafibrate treatment

In table 1 the weight of internal organs are showed. All organs analyzed in the DEXA group demonstrated a significant alteration in weight, except in epididymal and retroperitoneal fat compared to CTL (n=8, $P<0.05$). The liver weight increased in DEXA, BEZA and BEZA-DEXA groups by approximately 40%, 80% and 120% respectively, when compared to CTL group. In the BEZA-DEXA group only the heart weight remained unchanged compared to the DEXA group. While the kidney weights in the BEZA group showed a significant increase in relation to the CTL (n = 8, $P<0.05$).

**Effects of treatment with dexamethasone and bezafibrate on blood glucose, serum insulin, FFA, TG, HDL, T-Chol and hepatic glycogen content**

Table 2 shows the values measured in fasting animals. The DEXA group had increased FFA, TG, insulin levels and blood glucose, and decreased HDL cholesterol levels (n=8, $P<0.05$), but no change in the T-Chol levels compared to the CTL and BEZA groups. On the other hand, in the BEZA-DEXA group there was an increase in HDL cholesterol levels and a decrease in insulin, FFA and TG levels (n = 8, $P<0.05$) in relation to the DEXA group, whereas their glucose levels were equivalent to the DEXA group. In the BEZA-DEXA group the serum T-Chol levels remained unchanged. In the BEZA group no metabolic changes were observed when compared to the CTL.

Table 3 shows the values measured for fed animals. In the DEXA group the T-Chol levels demonstrated a significant increase when compared to the CTL and BEZA groups (n = 8, $P<0.05$) and HDL cholesterol levels remained unchanged. A rise in blood glucose levels which is caused by treatment with glucocorticoids

(P<0.05) did not occur in the BEZA-DEXA group. In the BEZA-DEXA group there was an increase in T-Chol levels compared to the BEZA.

The hepatic glycogen content in the fasting state (Fig. 3) increased in the DEXA group (n = 8, P<0.05) compared to the CTL. However, in the BEZA-DEXA group animals, there was a reduction (P<0.05) when compared to the DEXA group. In the BEZA group did not exhibit significant differences in the hepatic glycogen content in relation to CTL.

**Effects of treatment with dexamethasone and bezafibrate on Glucose Tolerance**

The DEXA treatment impaired the peripheral insulin resistance. The blood glucose values obtained in intraperitoneal glucose tolerance test (Fig.4A) carried out on the DEXA group were greater at all times during the experiment (n=8, P<0.05) compared to CTL. The BEZA group had unchanged blood glucose levels at all times during the experiment compared to CTL. The glucose levels of the BEZA-DEXA group were significantly higher at the15 and 30 minute intervals in relation the DEXA group. In addition, the area under the curve (AUC), obtained from ipGTT (Fig. 4B), in the DEXA group showed glucose intolerance (12575±344) compared to CTL (7529±671) and BEZA animals (8095±823) (n = 8, P<0.05). Furthermore, the BEZA-DEXA group (9094±714) improves glucose tolerance, when compared to the DEXA group.

The insulin concentration was significantly higher in the DEXA group (n = 8, P<0.05) than all other groups during the entire experimental period (Fig. 4C). Only at time interval 0 minutes did the BEZA-DEXA group show any significant increase in the serum insulin levels (n = 8, P<0.05) compared to CTL and BEZA. The insulin

AUC (Fig. 4D) was significantly increased in the DEXA group (5.89±1.25) in relation to CTL (1.01±0.14) and BEZA (0.91±0.02) groups (n = 8, P<0.05). In the BEZA-DEXA animals these values were reduced (2.47±1.23) compared to the DEXA group (n = 8, P<0.05)

**Effects of treatment with dexamethasone and bezafibrate on peripheral insulin sensitivity**

The values obtained during intraperitoneal insulin tolerance test are shown in Figure 5A. The DEXA group exhibited a decrease in insulin sensitivity demonstrated by a significant reduction in the constant rate for glucose disappearance (Kitt) values (13.0±0.76) compared to the CTL group (24.2± 2.3) and the BEZA group (32.0±4.9) (Fig.5B). The BEZA-DEXA animals demonstrated greater in insulin sensitivity with a significant increase in the Kitt (24.0±1.7) when compared to the DEXA group (n = 8, P <0.05).

**Glucose-induced insulin secretion in isolated islets**

The islet insulin content remained unchanged in all groups: CTL (160±30.5), DEXA (132±17.3), BEZA (167.87±23.5), BEZA-DEXA (107.87±11.6) ng/islet. As shown in Figure 6, the concentration of 2.8 mM glucose did not significant difference in insulin secretion between the groups. At the concentration of 16.7mM glucose, DEXA islets (15.46±0.6 ng/mL) and BEZA islets (16.11±1.0 ng/mL) had higher glucose-stimulated insulin secretion (GSIS) compared to the values in the CTL islets (7.80±0.3 ng/mL). The BEZA-DEXA islets showed a significant decrease in GSIS (10.94 ±0.4 ng/mL) when compared to DEXA and BEZA islets (n = 12, P<0.05).

**Protein expression of the GPR40 pathway in isolated islets**

To determine how the FFA stimulated the insulin secretion, we analyzed the protein expression of GPR40, PLCß1 and PKCζ. The protein expression of GPR40 in the BEZA-DEXA group (13031±767) was significantly higher compared to other groups (CTL, 9564±517; DEXA 9816±72 and BEZA 8495±739, n=4, $P<0.05$). However, the protein PLCß1 increased in animals from BEZA-DEXA (11513±841) only in relation to the DEXA group (8394 ± 217). When analyzed the protein expression PKCζ, observed an increase in the BEZA-DEXA group (10837±275) compared to other groups (CTL, 5075±668; DEXA, 4117±1424 and BEZA, 6472±234, n=4, $P<0.05$).

**DISCUSSION**

In this study, we investigated the contribution of the free fatty acids in dexamethasone-induced insulin resistance and insulin secretion in rats, by lipid-lowering therapy with bezafibrate.

The weight loss through dexamethasone treatment (Fig. 1) probably is due to the anorexigenic effects of insulin in the hypothalamus (Wood, et al., 1979), observed by a reduction of food intake (Fig. 2). However, the lower body weight loss in the BEZA-DEXA group compared to the DEXA group is supported by the increase in food intake on the last day of the dexamethasone-treatment, and by the reduction in the levels of insulin observed in this group. The administration of bezafibrate did not influence the food intake, neither body weight, unlike studies which reported a loss or gain in weight (Nakano et al., 2007, Santos et al., 2009). Counteracting the absolute weight loss in the DEXA and BEZA-DEXA groups, we observed a weight gain in both liver and kidney (table 1). The liver presented a higher weight gain among those

organs analyzed (table 1) in all groups compared to CTL, and this could be due to hepatomegaly promoted by activation of PPARα in rodents, but not in humans, as a result of parenchymal peroxisome proliferation through increased oxidative stress (Lee et al., 1995).

In the DEXA animals dyslipidemia was observed, a common feature among insulin resistant individuals (Yaney; Corney, 2003) with a marked increase in the levels of free fatty acids (FFA), triglycerides (TG), decrease in the levels of HDL cholesterol but did not change in the T-Chol levels in fasting rats (table 2). Bezafibrate treatment in normal fed rats exhibited only decreased TG levels (table 3). In the BEZA-DEXA animals there was a reduction in FFA, TG levels, an increase in HDL cholesterol levels and also the T-Chol levels remained unchanged (table 2) consistent with previous reports (Tenembaum et al., 2005). Bezafibrate is a pan (α, β/δ and γ) PPAR activator (Tenenbaum et al., 2007). PPARα is a major component of the cellular lipid homeostatic by regulating the expression of genes involved in lipid metabolism (Aoyama et al., 1998).

The DEXA group showed a significant increase in insulin values for both fasting (table 2) and fed rats (table 3), which did not efficiently maintain normoglycaemia because of insulin resistance. The BEZA-DEXA group had the insulin values decreased both in fasting (table 2) and fed conditions (table 3), which has been observed in other works (Sugden et al., 2003; Holness et al., 2005). Furthermore, the BEZA-DEXA group also experienced a decrease in the glycaemia of the fed rats, which could result in a reduction of insulin secretion. Kersten and cols. (1999) demonstrated that the PPARα is related to the adaptation of glucose in fasting state. However, in PPARα-null (-/-) there was no alteration in glycaemia compared to wild-type (+/+) (Nakajima et al., 2009) and the bezafibrate treatment in the IR

induced model produced no alterations in glycaemia (Jia et al., 2004; Pickavance et al., 2005).

Glucocorticoid-treatment stimulates the gluconeogenesis and the glucogenolysis. However, we observed a decrease in both pathways in the BEZA-DEXA group, confirmed by the improved glucose homeostasis and a decrease in the levels of serum insulin. Seung-Soon and cols. (2011) reported that in a type 2 diabetes animal model, PPARα could be activated either by free fatty acids or by glucocorticoid, causing up-regulation of gluconeogenic genes like hepatic glucose-6-phosphatase and of phosphoenolpyruvate carboxylase. They also demonstrated the synergistic effect of dexamethasone and a PPAR activator in the increased expression of these enzymes in the primary cultured hepatocytes.

Bezafibrate improves glucose tolerance (Fig 4) and insulin sensibility (Fig. 5) in dexamethasone-induced IR in rats. This fibrate is well known to restore normal insulin action in animal models of insulin resistance induced by high-saturated fat diets (Holness et al., 2003; Holness et al., 2005), high-fat high-sucrose diets (Santos et al., 2009), pregnancy (Sugden et al., 2003; Holness et al., 2006) and in Long-Evans Tokushima Fatty rats (Jia et al., 2004). Moreover, bezafibrate delayed the onset and decrease the incidence of type 2 diabetes in patients with impaired fasting glucose levels, and in obese patients over a long-term follow-up period (Tenenbaum et al., 2007; Flory, et al. 2009).

DEXA islets showed increase in the glucose-stimulated insulin secretion (GSIS) compared to CTL and BEZA-DEXA islets (Fig. 6). BEZA-DEXA islet restored the patterns of insulin secretion stimulated by glucose. Several models of IR demonstrated that the lipid lowering therapy reduced the GSIS (Lalloyer et al., 2006; Novelli et al., 2008). Already in BEZA islets there was a GSIS equivalent to DEXA

islets (Fig. 6) which is in agreement with previous reports (Yoshikawa et al., 2001; Shimomura et al., 2004; Ravnskjær et al., 2005). Shimomura and cols. (2004) described that PPARα and PPARγ may induce insulin secretion by direct inhibition of the ATP-sensitive potassium channel. Yoshikawa and cols. (2001) reported that islets incubated with glucose and PPARα activator had an increased mRNA expression of PPARα, acyl-CoA oxidase, carnitine-palmitoyltransferase-1 and pyruvate carboxylase, which could contribute to increased GSIS. On the other hand, neuronal, hormonal and metabolic factors can respond to an increase in GSIS of BEZA islet.

Studies have shown that FFA binding to GPR40 activates phosholipase C, resulting in the formation of inositol 1,4,5-triphosphate and diacylglycerol, thus activating protein kinase C (PKC) isoforms. PKC is responsible for stimulating the exocytosis of insulin secretory granules. Studies have shown that PKC ζ expression is regulated by LC-CoA (Yaney et al., 2000). Contrary to studies with GPR40, BEZA-DEXA animals showed reduced FFA and insulin levels, and increased protein content of the GPR40 pathway in isolated islets. According to Latour and cols. (2007), the GPR40 is necessary but not sufficient for fatty acid stimulation of insulin secretion *in vivo*. Thus, in the DEXA group there must be another mechanism responsible for lipid stimulation of insulin secretion. GPR40 is preferentially expressed in the pancreatic ß-cell. Although, there is evidence of the expression of GPR40 in the glucagon-producing pancreatic α-cells (Flodgren et a., 2007). The fatty acid linoleic acid activates GPR40 and phospholipase C resulting in increases in intracellular $Ca^{2+}$ and glucagon secretion (Wang et al., 2011).

These results indicate that treatment with bezafibrate improved ß-cell function and prevented the installation of insulin resistance imposed by dexamethasone.

Nevertheless, we are not able to say whether these results were due only to the circulating lipids. Augmented insulin secretion in the DEXA group appears to be unrelated to the activation of the GPR40. However, despite the reduction in insulin secretion, BEZA-DEXA islets showed activation of the GPR40 pathway, contrary to the literature.

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## FIGURE LEGENDS

**Fig.1** Average percent in body weight during the treatment. The groups DEXA and BEZA-DEXA demonstrated a significant reduction from the third day of the treatment with dexamethasone compared to other groups. Administration of Bezafibrate did not affect the weight of the animals. Data are means ± SEM. *Significant difference vs. CTL, ‡ *vs*. DEXA, † *vs.* BEZA and § *vs.* BEZA-DEXA. P <0.05, n = 8. ANOVA with *post test* Tukey.

**Fig. 2** Mean food intake during the days of treatment with dexamethasone. DEXA and BEZA-DEXA significantly reduced food intake from the second day of treatment compared to CTL and BEZA. However, the group BEZA-DEXA increased the food intake on the last day of treatment. The bezafibrate treatment does not influence the food intake compared to CTL group. Data are means ± SEM. *Significant difference vs. CTL, ‡ *vs*. DEXA, † *vs.* BEZA and § *vs.* BEZA-DEXA. P <0.05, n = 8. ANOVA with *post test* Tukey.

**Fig.3** Hepatic glycogen content. DEXA demonstrated a significant increase of hepatic glycogen content in relation to the CTL and BEZA groups. The BEZA-DEXA animals demonstrated a reduction when compared to values obtained from DEXA. Data are means ± SEM. *Significant difference vs. CTL, ‡ *vs*. DEXA, † *vs.* BEZA and § *vs.* BEZA-DEXA. P <0.05, n = 8. ANOVA with *post test* Tukey.

**Fig.4** Glucocorticoids-induced glucose intolerance is improved with bezafibrate treatment. (A) Blood glucose values obtained during ipGTT experiment in the DEXA

group demonstrated glucose intolerance. Its blood levels were greater at all times compared to the CTL group. There was an improvement in glucose tolerance of the BEZA-DEXA animals when compared to DEXA. The glucose levels in the BEZA animals were not significantly different at any time compared to CTL. (B) Area under the curve (AUC) of the ipGTT. (C) Insulin concentration in the DEXA group at all times demonstrated a significant increase compared to the other groups. The BEZA-DEXA group had significant increase in the serum insulin levels compared to CTL and BEZA only at time 0 minutes. (D) The insulin AUC values demonstrated a significant increase in the DEXA group compared to CTL and BEZA rats. In the BEZA-DEXA animals these values were reduced. Data are means ± SEM. *Significant difference vs. CTL, ‡ *vs*. DEXA, † *vs.* BEZA and § *vs.* BEZA-DEXA. P <0.05, n = 8. ANOVA with *post test* Tukey.

**Fig.5** Glucocorticoids-induced insulin resistance is improved by bezafibrate treatment. (A) Values of blood glucose obtained from ipITT. (B) Constant rate for glucose disappearance (Kitt). The DEXA group demonstrated insulin resistance compared to CTL and BEZA. The BEZA-DEXA animals showed improvement in insulin resistance when compared to the DEXA group. Data are means ± SEM. *Significant difference vs. CTL, ‡ *vs*. DEXA, † *vs.* BEZA and § *vs.* BEZA-DEXA. P <0.05, n = 8. ANOVA with *post test* Tukey.

**Fig.6** Static insulin secretion at 2.8 and 16.7mM glucose. At 2.8mM glucose, insulin secretion was no significantly different between the groups. At 16.7mM glucose concentration, the DEXA and BEZA islets secreted more insulin than the CTL islets. BEZA-DEXA islets had decreased insulin secretion when compared to the DEXA and

BEZA islets. Data are Means ± SEM. *Significant difference vs. CTL, ‡ *vs*. DEXA, † *vs.* BEZA and § *vs.* BEZA-DEXA. P <0.05, n = 12. ANOVA with *post test* Tukey.

**Fig. 7** Protein expression in pancreatic ß-cell from experimental groups. (A) G-protein-coupled receptor 40 (GPR40); (B) Phospholipase C ß1 (PLCß1); (C) Protein kinase C zeta (PKCζ). Data are means ± SEM. *Significant difference vs. CTL, ‡ *vs*. DEXA, † *vs.* BEZA and § *vs.* BEZA-DEXA. P <0.05, n = 4. ANOVA with *post test* Tukey.

Table 1: Metabolic-specific organ mass in the control (CTL) group, dexamethasone-treated (DEXA), bezafibrate-treated (BEZA), and bezafibrate and dexamethasone-treated (BEZA-DEXA) rats.

| (g/Kg BW) | CTL | DEXA | BEZA | BEZA-DEXA |
|---|---|---|---|---|
| Liver | 30.23 ±0.66 | 42.12 ±1.55 * | 54.68 ±2.03 * ‡ | 67.02 ±2.23 * ‡ † |
| Heart | 3.50 ±0.12 | 4.01 ±0.06 * † | 3.46 ±0.13 | 3.88 ±0.10 |
| Spleen | 1.92 ±0.06 | 1.23 ±0.03 * † | 1.99 ±0.07 | 1.31 ±0.06 * † |
| Kidney | 6.99 ±0.12 | 8.10 ±0.15 * | 7.66 ±0.13 * | 8.29 ±0.26 * |
| Epididymal | 10.70 ±0.64 | 13.40 ±1.05 | 10.73 ±1.14 | 11.50 ±1.05 |
| Retroperitoneal | 11.66 ±1.00 | 11.10 ±1.49 | 9.72 ±1.38 | 10.67 ±1.47 |

**Note:** Data are means ± SEM * Significant difference *vs*. CTL; ‡ *vs*. DEXA and † *vs*. BEZA and § vs. BEZA-DEXA. P <0.05, n = 8. ANOVA with *post test* Tukey.

Table 2: Blood Glucose and Serum Parameters obtained in fasting rats in the control (CTL) group, dexamethasone-treated (DEXA), bezafibrate-treated (BEZA), and bezafibrate and dexamethasone-treated (BEZA-DEXA) rats.

| | **GLUCOSE** | **FFA** | **TG** | **HDL** | **T-Chol** | **INSULIN** |
|---|---|---|---|---|---|---|
| | (mg/dL) | (mM) | (mg/dL) | (mg/dL) | (mg/dL) | (ng/mL) |
| CTL | 91.6 ± 2.5 | 0.42 ± 0.1 | 66.6 ± 13.6 | 33.8 ± 2.5 | 44.9 ± 6.2 | 2.01 ± 0.2 |
| DEXA | 129.0 ± 14.4 * | 1.09 ± 0.15 * † | 242.5 ± 19.3 * † | 11.6 ± 2.2 * † | 51.5 ± 7.8 | 12.16 ± 1.2 * † |
| BEZA | 114.4 ± 2.2 | 0.44 ± 0.11 | 52.5 ± 6.5 | 26.5 ± 2.2 | 38.2 ± 4.4 | 1.84 ± 0.1 |
| BEZA-DEXA | 123.8 ± 4.6 * | 0.33 ± 0.07 ‡ | 103.0 ± 9.2 ‡ | 40.6 ± 6.4 ‡ | 43.7 ± 5.4 | 3.13 ± 0.3 ‡ |

**Note:** Data are means ± SEM * Significant difference *vs*. CTL; ‡ *vs*. DEXA and † *vs*. BEZA. P <0.05, n = 8. ANOVA with *post test* Tukey.

Table 3: Blood Glucose and Serum Parameters obtained in fed rats in the control (CTL) group, dexamethasone-treated (DEXA), bezafibrate-treated (BEZA), and bezafibrate and dexamethasone-treated (BEZA-DEXA) rats.

| | GLUCOSE | FFA | TG | HDL | T-Chol | INSULIN |
|---|---|---|---|---|---|---|
| | (mg/dL) | (mM) | (mg/dL) | (mg/dL) | (mg/dL) | (ng/mL) |
| CTL | 123.2 ± 7.2 | 0.72 ± 0.12 | 160.9 ± 17.7 | 52.9 ± 3.1 | 71.2 ± 8.9 | 6.24 ± 0.4 |
| DEXA | 313.2 ± 38.0 * † | 1.63 ± 0.14 * † | 230.8 ± 17.6 * † | 45.2 ± 3.8 | 94.9 ± 4.1 * † | 28.0 ± 1.6 * † |
| BEZA | 124.0 ± 2.6 | 0.59 ± 0.17 | 64.5 ± 2.72 * | 48.4 ± 4.7 | 59.7 ± 2.3 | 3.13 ± 0.2 |
| BEZA-DEXA | 137.4 ± 3.7 ‡ | 0.97 ±0.1 ‡ | 117.7 ± 15.5 ‡ | 62.2 ± 4.0 ‡ | 87.4 ± 4.7 † | 18.31 ± 1.7 * ‡† |

**Note:** Data are means ± SEM * Significant difference *vs*. CTL; ‡ *vs*. DEXA and † *vs*. BEZA and § *vs*. BEZA-DEXA. P <0.05, n = 8. ANOVA with *post test* Tukey.

**Figure 1.**

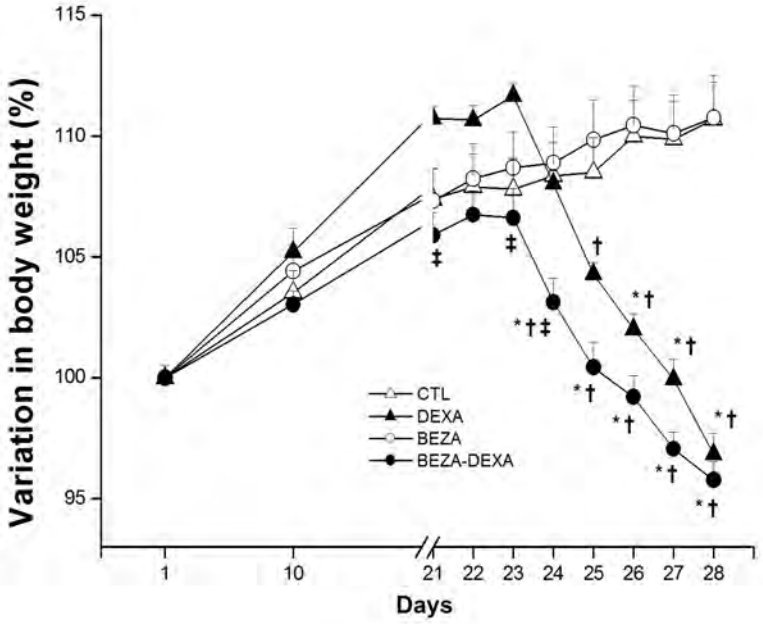

Variation in body weight (%)
115
110
105
100
95
1
10
21
22
23
24
25
26
27
28
Days
CTL
DEXA
BEZA
BEZA-DEXA

**Figure 2.**

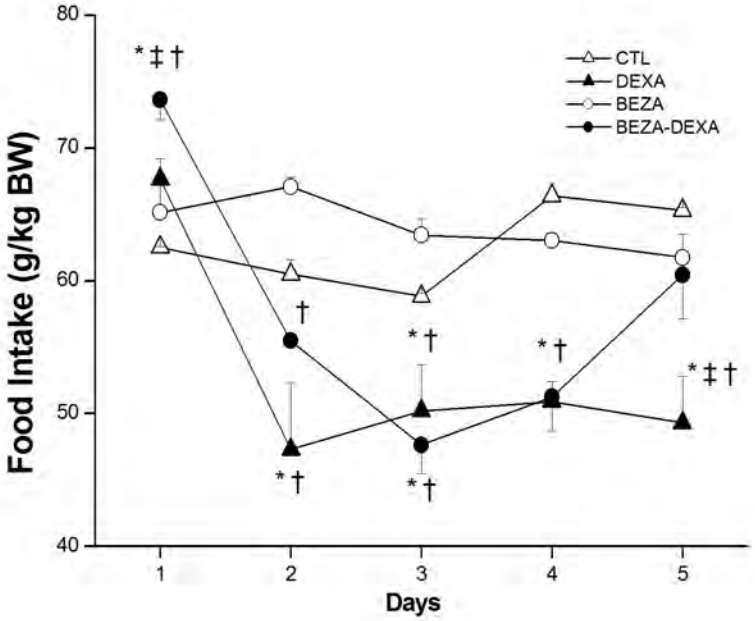

80
70
60
50
40
Food Intake (g/kg BW)
1
2
3
4
5
Days
CTL
DEXA
BEZA
BEZA-DEXA
*‡†
†
*†
*†
*†
*‡†
*†
*†

**Figure 3.**

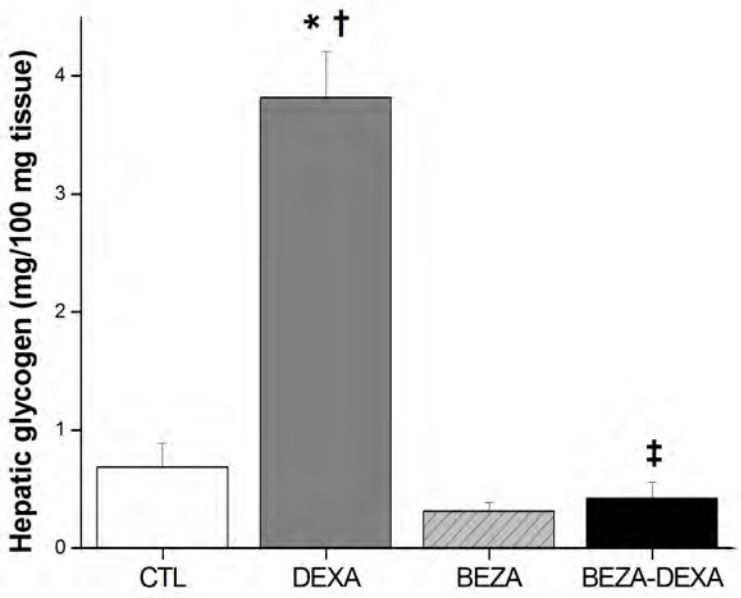

Hepatic glycogen (mg/100 mg tissue)
0
1
2
3
4
* †
‡
CTL
DEXA
BEZA
BEZA-DEXA

**Figure 4.**

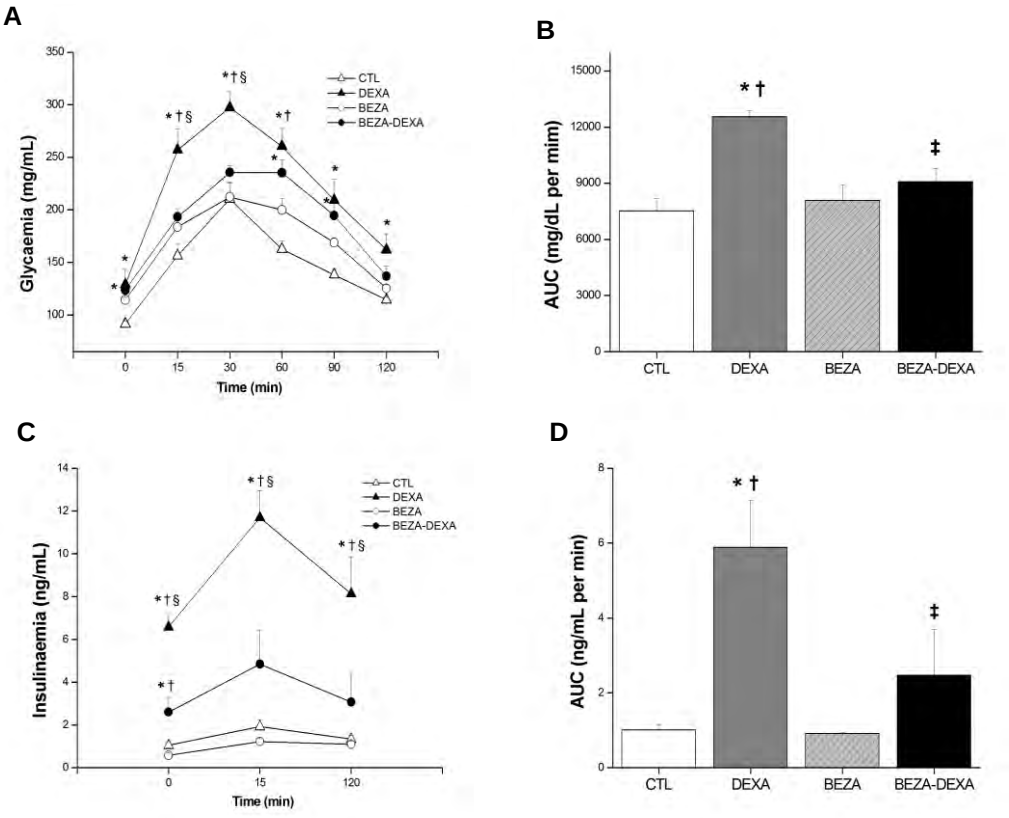

A
Glycaemia (mg/mL)
Time (min)
CTL
DEXA
BEZA
BEZA-DEXA
B
AUC (mg/dL per mim)
CTL
DEXA
BEZA
BEZA-DEXA
C
Insulinaemia (ng/mL)
Time (min)
CTL
DEXA
BEZA
BEZA-DEXA
D
AUC (ng/mL per min)
CTL
DEXA
BEZA
BEZA-DEXA

**Figure 5.**

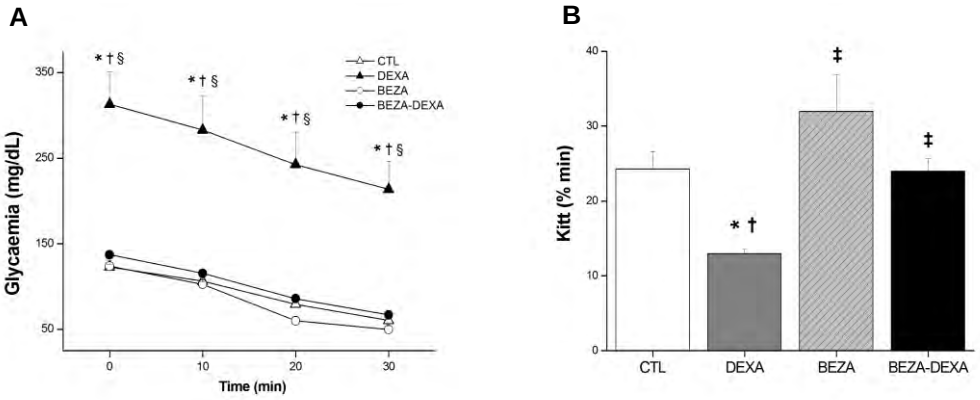


**Figure 6.**

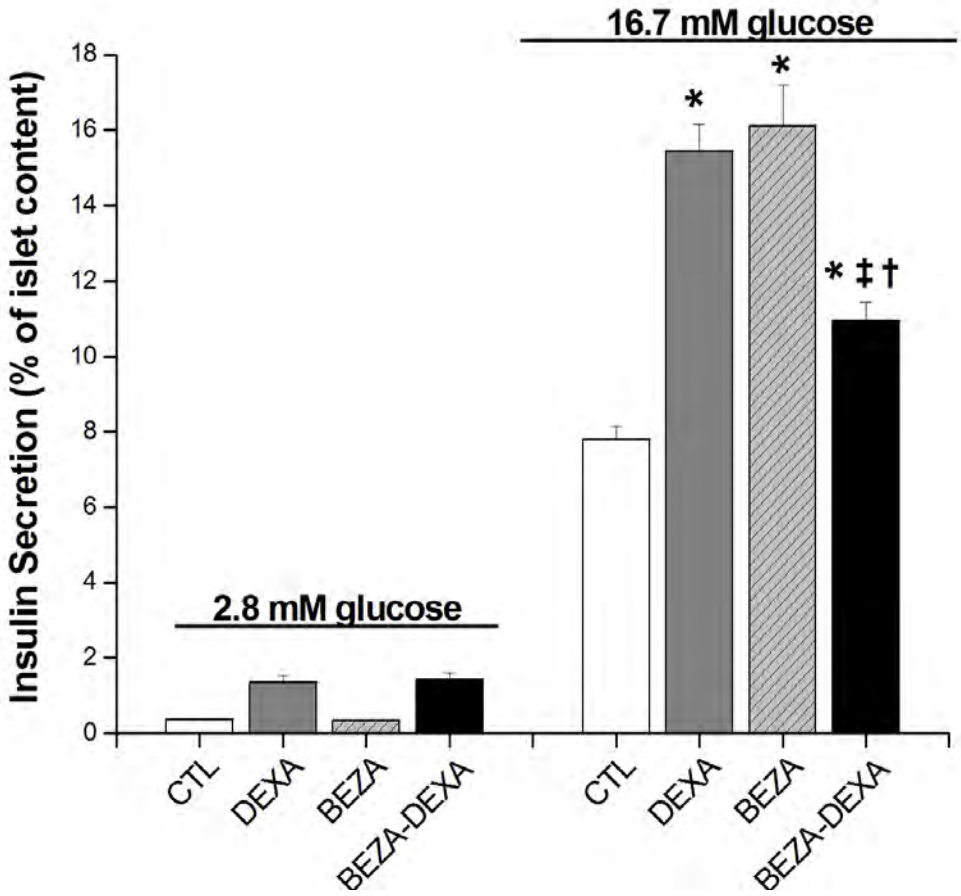
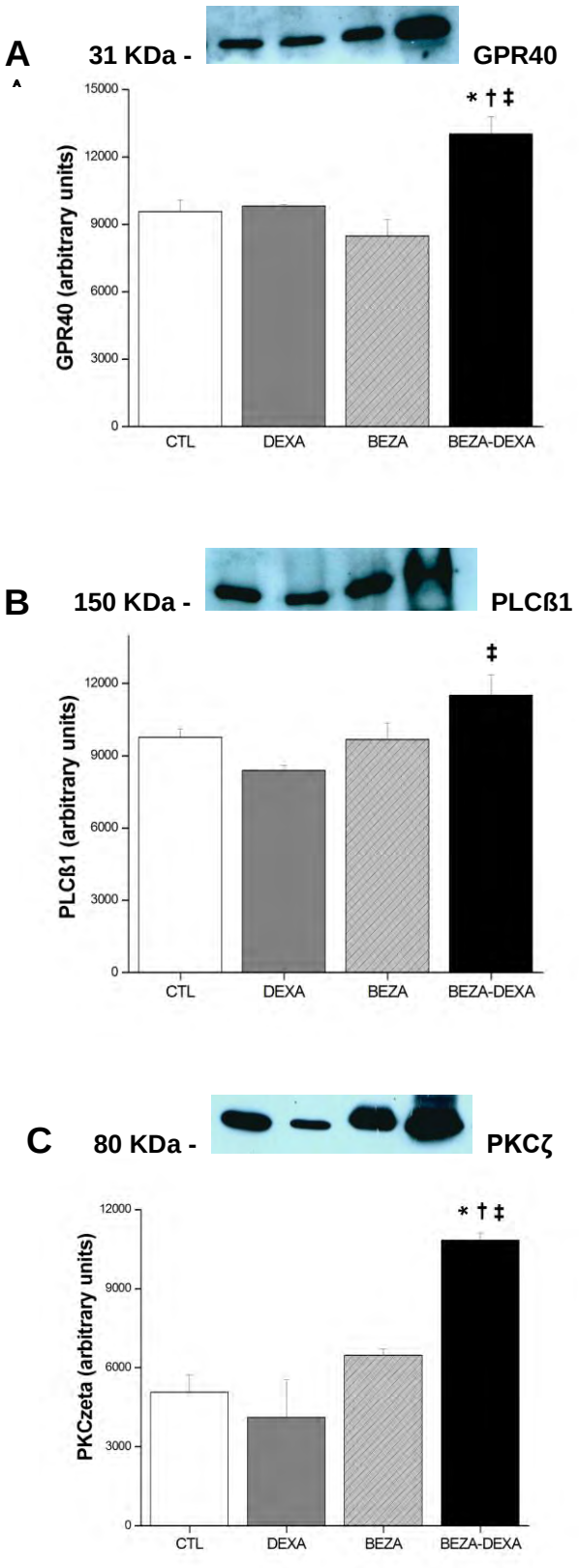

16.7 mM glucose
2.8 mM glucose
Insulin Secretion (% of islet content)
18
16
14
12
10
8
6
4
2
0
*
*
* ‡ †
CTL
DEXA
BEZA
BEZA-DEXA
CTL
DEXA
BEZA
BEZA-DEXA

**Figure 7.**


A
31 KDa -
GPR40
GPR40 (arbitrary units)
15000
12000
9000
6000
3000
0
* † ‡
CTL
DEXA
BEZA
BEZA-DEXA
B
150 KDa -
PLCß1
PLCß1 (arbitrary units)
12000
9000
6000
3000
0
‡
CTL
DEXA
BEZA
BEZA-DEXA
C
80 KDa -
PKCζ
PKCzeta (arbitrary units)
12000
9000
6000
3000
0
* † ‡
CTL
DEXA
BEZA
BEZA-DEXA

## *Conclusão*

O tratamento prévio com bezafibrato em ratos induzidos a resistência à insulina por dexametasona causa:

- Redução nos níveis séricos de lipídios, glicose e insulina;
- Redução no conteúdo de glicogênio hepático;
- Melhora na resistência periférica à insulina e na função da célula ß;
- Aumento na expressão da via de sinalização do GPR40.

Assim, o tratamento com bezafibrato melhorou a função das células ß e impediu a indução de resistência à insulina pelo tratamento com dexametasona, mas os mecanismos não são conhecidos. O aumento na secreção de insulina em DEXA aparentemente não está relacionado com a ativação do GPR40. Contrariando a literatura, apesar da redução na secreção de insulina, as ilhotas dos animais BEZA-DEXA apresentaram ativação da via do GPR40.

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