Universidade Federal do Rio Grande do Sul

Programa de Pós-Graduação em Ciências Médicas: Endocrinologia

Exendin-4 na prevenção de danos teciduais decorrentes da morte encefálica e no controle bioenergético da célula β pancreática

Tese de doutorado

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- **Artigo original 1**: "Exendin-4 protects rat islets against loss of viability and function induced by brain death" (publicado na revista *Molecular and Cellular Endocrinology* em 2015).

- **Artigo original 2**: "Exendin-4 attenuates brain death-induced liver damage in the rat" (publicado na revista *Liver Transplantation* em 2015).

- **Artigo original 3**: GLP-1 receptor signaling promotes β-cell glucose metabolism via mTOR-dependent HIF-1α activation" (submetido para publicação na revista *Diabetes*).

Lista de abreviaturas para a Introdução

ADP	*adenosine diphosphate*
ATP	*adenosine triphosphate*
cAMP	cyclic adenosine monophosphate
cAMP GEF	c*AMP-regulated guanine nucleotide exchange factor*
CICR	*calcium-induced calcium release*
CREB	*cAMP response element-binding protein*
DM	diabetes mellitus
DM1	diabetes mellitus tipo 1
DM2	diabetes mellitus tipo 2
DPP-4	*Dipeptidil-peptidase-4*
EPAC	*Exchange protein activated by cAMP*
GIP	*Glucose-dependent insulinotropic polypeptide*
GLP-1	*Glucagon-like peptide-1*
GLP-1R	*Glucagon-like peptide-1 receptor*
GLUT2	*Glucose transporter 2*
HIF-1	*Hypoxia-inducible factor 1*
IL-1β	*Interleukin-1 beta*
Kv	*voltage-gated potassium channel*
ME	morte encefálica
mTOR	*mammalian Target of Rapamycin*
NAFLD	*nonalcoholic fatty liver disease*
NASH	*nonalcoholic steatohepatitis*
PKA	*Protein kinase A*
VDCCs	*voltage dependent calcium channels*

SUMÁRIO

RESUMO

A reposição de ilhotas pancreáticas é uma forma eficaz de se restabelecer a homeostase glicêmica em pacientes com diabetes mellitus tipo 1 com controle metabólico instável ("DM1 lábil"). Entretanto, ao longo do processo de isolamento, a partir do pâncreas do doador em morte encefálica (ME) e posterior enxerto no receptor, perdas importantes, tanto no número quanto na qualidade das ilhotas, limitam a eficácia do procedimento de transplante. Frequentemente, transplantes sequenciais de dois ou até três pâncreas são necessários para se atingir a independência de insulina exógena. Mesmo que centros transplantadores já reportem boas taxas de sucesso, utilizando apenas um doador por paciente, a escassez de doadores ainda é um forte limitador dessa terapêutica, dificultando a adoção da reposição de ilhotas como prática clínica de rotina para o tratamento de pacientes com DM1 lábil.

Entre os fatores responsáveis pela perda das ilhotas, pode-se destacar o intenso estresse inflamatório produzido pela ME do doador. Semelhantemente ao que ocorre com ilhotas pancreáticas, estudos clínicos e experimentais evidenciam que a ME induz danos teciduais irreversíveis em vários outros órgãos e tecidos destinados a transplante, tais como o coração, pulmões, rins e fígado. Órgãos provenientes de doadores vivos, em geral, resultam em desfechos pós-transplante mais favoráveis quando comparados às taxas de sucesso obtidas com órgãos derivados de doadores cadavéricos.

Estudos recentes demonstram que a Exendin-4, um análogo do *glucagon-like peptide-1* (GLP-1), possui propriedades anti-inflamatórias, pró-proliferativas e de redução da apoptose de células β pancreáticas. Interessantemente, esse mesmo

análogo também apresentou efeitos protetores em diversos modelos animais de doenças hepáticas. Sendo assim, nós hipotetizamos que a administração dessa droga a doadores cadavéricos poderia amenizar os danos causados pela ME nas ilhotas pancreáticas e no tecido hepático, preservando a qualidade desses tecidos para uso em transplantes.

A fim de testar a hipótese sugerida, desenvolveram-se dois estudos em modelo murino de ME. A administração de Exendin-4 a animais com ME foi avaliada em relação a diversos parâmetros de dano e qualidade teciduais, além de marcadores inflamatórios e expressão gênica de genes ligados a inflamação e estresse. Grupos de animais que não sofreram ME ou que sofreram ME mas não receberam a droga foram utilizados como controles nesses experimentos.

Os resultados gerados indicam que a administração de Exendin-4 a ratos em ME melhora a viabilidade e a função de ilhotas pancreáticas isoladas. Isso foi acompanhado por uma redução na expressão gênica da citocina pró-inflamatória *Interleukin-1 beta* (Il1b) no tecido pancreático. Além disso, o tratamento com Exendin-4 também modulou a expressão de genes que codificam para proteínas de controle do estresse oxidativo celular, a *superoxide dismutase-2* (Sod2) e a *uncoupling protein-2* (Ucp2). Genes relacionados ao estresse do retículo endoplasmático, *C/EBP Homologous Protein* (Chop) e Heat Shock 70kDa Protein 5 (Hspa5), também conhecido como *immunoglobulin heavy-chain binding protein* (BiP), tiveram suas expressões reduzidas em ilhotas isoladas de animais tratados. Recentemente, tem sido demonstrado que o estresse do retículo endoplasmático participa no mecanismo de morte celular da célula β induzida por citocinas. Assim, nós sugerimos que o efeito protetor da Exendin-4 contra os danos causados pela ME provavelmente se dê através de uma diminuição da inflamação e do estresse oxidativo

no ambiente pancreático, o que se traduz em uma menor ativação do estresse do retículo endoplasmático na célula β, consequentemente, reduzindo a morte celular nas ilhotas isoladas.

Em ressonância com os efeitos benéficos observados nas ilhotas, animais tratados também apresentaram níveis reduzidos de marcadores de dano hepático circulantes, além de uma diminuição significativa nas taxas de apoptose no tecido hepático. Os resultados aqui apresentados, portanto, sugerem que a administração de Exendin-4 a doadores cadavéricos de múltiplos órgãos tem o potencial para minimizar os efeitos deletérios causados pela ME nas ilhotas pancreáticas e no fígado.

O GLP-1, uma incretina gastrointestinal secretada pelas células L do intestino delgado, estimula a secreção de insulina dependente de glicose nas células β pancreáticas. Estudos recentes demonstram que o GLP-1 é capaz de proteger as células β do estresse oxidativo e dos danos inflamatórios, os quais são considerados fatores importantes não apenas na mediação dos danos teciduais induzidos pela ME, mas também na patogênese do diabetes mellitus tipo 2 (DM2). Tal efeito estimulatório dependente de glicose pode ser explorado como uma estratégia terapêutica alternativa para o tratamento de pacientes com DM2, por reestabelecer a homeostase glicêmica e eliminar o risco de hipoglicemia associada à injeção exógena de insulina. Recentemente, vários ensaios clínicos confirmaram os efeitos benéficos de análogos do GLP-1, que incluem um melhor controle glicêmico e perda de peso, resultando na aprovação de tais análogos para o tratamento clínico do DM2. No entanto, os mecanismos moleculares que medeiam a estimulação da secreção de insulina pelo GLP-1 não são totalmente conhecidos. Isso gera a necessidade do desenvolvimento de estudos bioquímicos detalhados para elucidação das vias de transdução de sinal intracelulares responsáveis pela execução dos efeitos

estimulatórios do GLP-1 na secreção de insulina.

Devido à dependência de glicose para a ativação do seu efeito estimulatório, nós hipotetizamos que o GLP-1 possa atuar na regulação da atividade bioenergética de metabolismo da glicose na célula β. Utilizando-se da linhagem celular clonal secretora de insulina, BRIN-BD11 e ilhotas isoladas de camundongos, nós demonstramos que a sinalização através do receptor do GLP-1 (GLP-1R) promove a glicólise e, consequentemente, aumenta o conteúdo de ATP intracelular. Os dados deste estudo sugerem que isso provavelmente seja mediado pela ativação da *mammalian Target of Rapamycin* (mTOR), resultando na acumulação do fator de transcrição *Hypoxia-inducible factor 1 alpha* (HIF-1α), que por sua vez promove um aumento na expressão gênica de genes que codificam para enzimas da via glicolítica. Sugere-se que essa indução na taxa glicolítica possa reforçar o acoplamento metabólico entre estímulo e secreção, resultando no aumento da secreção de insulina dependente de glicose mediada por GLP-1.

ABSTRACT

Replacement of pancreatic islets is an effective way to restore glucose homeostasis in patients with type 1 diabetes mellitus with unstable metabolic control ("T1DM labile"). However, along the isolation procedure from brain dead organ donors and later recipient engraftment, significant losses, both in number and quality of islets take place, limiting the effectiveness of islet transplantation as a whole. Often, sequential transplants of two or three donors are necessary in order to achieve exogenous insulin independence. Even with recent advances and transplant centers already reporting good success rates using only one donor per patient, the shortage of donors is still a strong limitation of this therapy, hindering the adoption of islets reposition as routine clinical practice for the treatment of patients with T1DM labile. Several factors are responsible for islets loss, including intense inflammatory stress produced by the donor's brain death (BD). Similarly to what happens with pancreatic islets, clinical and experimental studies show that BD induces irreversible tissue damage in several other organs and tissues destined for transplantation, such as heart, lungs, kidneys and liver. In general, organs from living donors result in more favorable post-transplant outcomes when compared to organs procured from cadaveric donors.

Recent studies indicate that Exendin-4, a glucagon-like peptide-1 (GLP-1) analog, has anti-inflammatory, proliferative and anti-apoptotic properties in pancreatic β cells. Interestingly, this analog has also shown protective effects in several animal models of liver disease. Thus, we hypothesized that administration of this drug to cadaveric donors could mitigate damage caused by BD in the pancreatic

islets and liver tissue. Such treatment could preserve islets and liver quality for use in transplantation and possibly improve outcomes.

In order to test the suggested hypothesis, we developed two studies using a rat model of BD. Administration of Exendin-4 to animals that underwent experimental BD was evaluated against various parameters of tissue damage and quality, as well as inflammatory markers and gene expression of genes linked to inflammation and stress. Groups of animals that did not undergo BD or that underwent BD, but have not been given the drug were used as controls in these experiments.

Results generated in these studies indicate that administration of Exendin-4 to brain dead rats improves the viability and function of isolated pancreatic islets. This was accompanied by a decrease in gene expression of the pro-inflammatory cytokine Interleukin-1 beta (Il1b) in the pancreatic tissue. In addition, treatment with Exendin-4 also modulated the expression of genes encoding cellular oxidative stress control proteins, the superoxide dismutase-2 (Sod2), and uncoupling protein-2 (Ucp2). Genes related to stress of the endoplasmic reticulum (ER), C/EBP Homologous Protein (Chop) and Heat Shock 70kDa Protein 5 (Hspa5), also known as heavy-chain binding protein immunoglobulin (BiP), were found to be reduced in islets isolated from treated animals. Recently, ER stress has been shown to participate in the mechanism of β cell death induced by pro-inflammatory cytokines. Thus, we suggest that the protective effect of Exendin-4 against damage caused by BD probably occurs through a decrease in inflammation and oxidative stress in the pancreatic environment, which translates into a lower activation of ER stress in the β cells, hence reducing cell death of isolated islets.

In resonance with the beneficial effects observed in islets, treated animals also showed reduced levels of circulating liver damage markers, and a significant decrease

in the rate of apoptosis in the liver. Our results, therefore, suggest that administration of Exendin-4 to cadaveric organ donors has the potential to minimize the deleterious effects caused by BD in the pancreatic islets and liver.

GLP-1, a gastrointestinal incretin secreted by the L-cells of the small intestine, stimulates insulin secretion in a glucose-dependent manner from pancreatic β cells. Recent studies have shown that GLP-1 is able to protect β cells from oxidative stress and inflammatory damage, which are considered important factors not only in mediating tissue damage induced by BD, but also in the pathogenesis of type 2 diabetes mellitus (T2DM). Such glucose-dependent stimulatory effect can be exploited as an alternative therapeutic strategy for the treatment of patients with T2DM in order to reestablish glucose homeostasis and to eliminate the risk of hypoglycemia associated with exogenous insulin injection. Recently, several clinical trials have confirmed the beneficial effects of GLP-1 analogs, which include better glycemic control and weight loss, resulting in the approval of such analogs for the clinical treatment of T2DM. However, the molecular mechanisms that mediate stimulation of insulin secretion by GLP-1 are not fully characterized. Thus, detailed biochemical studies aiming at elucidating intracellular signal transduction pathways responsible for the execution of GLP-1 stimulatory effects on insulin secretion are of high interest.

Due to glucose-dependence for activation of its stimulatory effect, we hypothesized that GLP-1 may act in the regulation of β cell bioenergetics and glucose metabolism. Using the clonal rat insulin-secreting cell line BRIN-BD11, and isolated mouse islets, we have demonstrated that signaling via the GLP-1 receptor (GLP-1R) promotes glycolysis and consequently increases intracellular ATP content. Our data suggest that this is likely mediated by activation of the mammalian Target of

Rapamycin (mTOR), resulting in the accumulation of the transcription factor Hypoxia-inducible factor 1 alpha (HIF-1α), which, in turn, promotes gene expression of genes that encode for glycolytic enzymes. We suggest that such increase in the glycolytic rate strengthens coupling between metabolic stimulus and secretion, ultimately resulting in exacerbated glucose-induced insulin secretion.

1. INTRODUÇÃO

1.1 Diabetes mellitus tipo 1 e o transplante de ilhotas

O diabetes mellitus (DM) é um grupo de doenças metabólicas caracterizadas por uma falha na homeostase da glicose, levando à hiperglicemia crônica [1]. Tal descontrole metabólico é causado por defeitos na secreção e/ou ação da insulina [2] De acordo com a intensidade e com o tempo de exposição à hiperglicemia, ocorrem lesões estruturais no endotélio vascular e no tecido nervoso. Estas causam disfunções e falhas de vários órgãos e tecidos, levando ao aparecimento das complicações crônicas microangiopáticas (retinopatia diabética, doença renal do diabetes e neuropatia diabética) e macroangiopáticas (infarto agudo do miocárdio, acidente vascular cerebral e gangrena) do DM [2]. Tais doenças secundárias contribuem para morbidade e mortalidade associadas ao DM e requerem elevados custos para tratamento.

Representando 5% a 10% dos casos de DM, o diabetes mellitus tipo 1 (DM1) é caracterizado pela destruição autoimune das células pancreáticas produtoras de insulina (células β das ilhotas de Langerhans). Portanto, os pacientes com DM1 requerem administração exógena de insulina para sobreviver [1]. De fato, o tratamento com insulina é a terapia indicada para pacientes diabéticos tipo 1 e comprovadamente diminui os riscos de complicações secundárias [3].

Apesar disso, a hipoglicemia associada a esse tipo de tratamento constitui um perigoso efeito colateral, especialmente em pacientes com “DM1 lábil”. O DM1 lábil é definido por amplas variações da glicemia capilar ao longo do dia, tipicamente maiores do que 200 mg/dl e que interfiram na qualidade de vida do paciente, ou por

hipoglicemias graves sem sintomas adrenérgicos de alerta. Para esses casos, o transplante de pâncreas como órgão inteiro consegue restaurar a normoglicemia e apresenta uma boa taxa de sobrevivência, tanto do paciente quanto do enxerto [4]. Entretanto, o procedimento está associado a riscos e morbidade de uma cirurgia de grande porte.

Desde a década de 1990, quando o primeiro transplante de ilhotas em humanos foi realizado com sucesso [5], que o transplante de ilhotas pancreáticas se tornou uma alternativa terapêutica promissora para pacientes com DM1 lábil. Contudo, nos anos seguintes, o transplante de ilhotas passou a ser realizado em diversos centros ao redor do mundo, e as taxas de sucesso reportadas não atingiram níveis satisfatórios. Sendo assim, o transplante de pâncreas continua sendo o tratamento de escolha para o "DM lábil" [6, 7].

O protocolo para isolamento e transplante de ilhotas com maior sucesso clínico até o momento foi desenvolvido na universidade de Edmonton por Shapiro e colaboradores [8]. Nesse estudo, todos os sete pacientes transplantados permaneceram independentes de insulina um ano após o transplante. A longo prazo, entretanto, cinco anos após o transplante original, somente 10% dos pacientes continuaram independentes de insulina. Apesar disso, a instabilidade metabólica e as hipoglicemias graves mantiveram-se satisfatoriamente atenuadas [9]. O transplante de ilhotas pode ser, então, considerado satisfatório, se parâmetros mais realistas, tais como prevenção de hipoglicemias graves e estabilização dos níveis de glicose, forem levados em conta [10]. Mais recentemente, os diferentes centros transplantadores vêm desenvolvendo melhorias nas técnicas de preparação de ilhotas, além de regimes de imunossupressão mais modernos, com isso atingindo desfechos progressivamente melhores [11]. Dados publicados recentemente pela *Collaborative Islet Transplant Registry* (CITR)

revelaram que aproximadamente 50% de todos os pacientes que receberam transplantes de ilhotas entre 2007 e 2010 permanecem independentes de insulina três anos após o transplante [12].

Provavelmente, a maior desvantagem do transplante de ilhotas seja o fato de que um controle glicêmico adequado pós-transplante exige que um grande número de ilhotas seja transplantado. Por volta de 10.000 equivalentes de ilhota por quilograma de peso corporal do paciente. Transplantes sequenciais de dois ou até três pâncreas são muitas vezes necessários para se atingir a independência de insulina, e a escassez de órgãos para transplante é um forte limitador dessa terapêutica [13-15]. Mesmo assim, alguns centros, como o *Schulze Diabetes Institute*, em Minneapolis, EUA, obtiveram sucesso ao atingir percentuais satisfatórios de independência insulínica, fazendo transplantes de um único doador [16]. O mesmo centro, recentemente, publicou dados demonstrando graus de sucesso similares entre transplante de ilhotas e transplante de pâncreas como órgão inteiro, com aproximadamente 50% de independência insulínica cinco anos após o transplante [17]. A perda de ilhotas ao longo dos procedimentos de isolamento e implantação, além da rejeição pelo receptor, estão entre os principais alvos de pesquisa com o objetivo de melhoria do processo de transplante como um todo [4]. Em função disso, atualmente, busca-se aprimorar tais procedimentos a fim de se atingir o máximo aproveitamento de ilhotas por pâncreas doado [18].

1.2 A morte encefálica do doador e seus efeitos deletérios

Ao longo do procedimento de remoção e estocagem dos pâncreas provenientes de doadores cadavéricos, as ilhotas são submetidas a diversos fatores, que, somados, induzem a extensiva morte celular. Entre eles, destacam-se: isquemia fria, estresse

oxidativo e forças de cisalhamento que agem sobre o órgão, além do processo de digestão necessário ao isolamento das ilhotas [19, 20]. Soma-se a esses fatores a própria natureza das células β, que, por estarem em avançado estágio de diferenciação, possuem baixa capacidade regenerativa [21]. Por último, mas não menos importante, destaca-se o intenso estresse inflamatório produzido pela morte encefálica (ME) do doador, resultando em lesão tecidual e redução da função e da sobrevida dos enxertos [22].

De fato, na imensa maioria dos estudos de transplante de órgãos, animais vivos são utilizados como doadores, ao contrário da prática clínica, na qual, em geral, os doadores são pacientes em ME [23]. O impacto e os efeitos deletérios que a ME exerce sobre a qualidade dos órgãos para transplante têm sido estudados desde a década de 1990. Tais efeitos negativos para órgãos como o coração, rins, fígado, pulmões e mesmo para as ilhotas pancreáticas vêm sendo investigados [24-29]. Acredita-se que alterações hemodinâmicas, neuro-humorais e imunológicas decorrentes da ME sejam os principais fatores que alteram a qualidade dos órgãos [28]. Em um estudo recente do nosso grupo, demonstrou-se que a ME induz inflamação do tecido pancreático humano [30]. Inflamação, através da ação de citocinas pró-inflamatórias, reconhecidamente promove a morte celular da célula β e participa na patogênese tanto do DM1 quanto do diabetes mellitus tipo 2 (DM2) [31]. Assim, fica claro que o combate aos efeitos deletérios causados pela ME possui imenso potencial de utilização na melhoria da qualidade dos diferentes órgãos e tecidos destinados a transplante, incluindo o fígado e as ilhotas pancreáticas.

1.3 Glucagon-like peptide-1 e seus análogos

Recentemente, um importante alvo de pesquisas na área do DM tem sido o *glucagon-like peptide-1* (GLP-1), um hormônio peptídico liberado principalmente pelas células L do intestino delgado, quando estimuladas pela presença de nutrientes no lúmen do intestino. Sua liberação na corrente sanguínea estimula a secreção de insulina dependente de glicose e inibe a secreção de glucagon [32, 33]. Como resultado, a produção de glicose hepática e a concentração sanguínea da mesma caem [34, 35]. A habilidade do GLP-1 de modular a secreção de insulina e glucagon é preservada em pacientes com DM2 [36, 37]. Sendo assim, GLP-1 efetivamente reduz a hiperglicemia nesses pacientes [38-40]. Entretanto, a forma selvagem do GLP-1 é degradada rapidamente na circulação pela peptidase plasmática *dipeptidil-peptidase-4* (DPP-4). A clivagem proteolítica pela DPP-4 faz com que a meia-vida do GLP-1 seja inferior a dois minutos na circulação após a sua secreção inicial pelas células L intestinais [41]. Assim, a indústria farmacêutica tem investido no desenvolvimento de análogos do GLP-1 resistentes à degradação pela DPP-4. Ensaios clínicos randomizados recentes têm evidenciado que os análogos do GLP-1 melhoram o controle glicêmico e a função da célula β em pacientes com DM2 [42-45]. Isso levou à aprovação da Exendin-4 (Exenatida) e da Liraglutida, dois análogos estáveis do GLP-1, para o tratamento clínico do DM2 em vários países, inclusive o Brasil.

A Exendin-4, além do seu uso terapêutico devido à estimulação na secreção de insulina, demonstra propriedades citoprotetoras para ilhotas pancreáticas [46, 47]. A estimulação do receptor do GLP-1 (GLP-1R) produz efeitos diretos nas células β, resultando em aumento da regeneração celular, proliferação e redução da apoptose, tanto em modelos animais como em ilhotas humanas isoladas [48, 49]. Assim, seu potencial como agente citoprotetor no transplante de ilhotas para pacientes com DM1

foi sugerido e testado clinicamente [50, 51]. Os resultados positivos desses estudos clínicos indicam que, em breve, a Exendin-4 poderá ser incluída como parte do tratamento básico dado a pacientes com DM1 transplantados. Além das propriedades já citadas, a Exendin-4 possui também atividade anti-inflamatória em linhagens de células β e ilhotas pancreáticas isoladas. Alguns estudos mostram a ação protetora da Exendin-4 contra a atividade pró-inflamatória de citocinas como a *Interleukin-1 beta* (IL-1β) [52, 53]. Um estudo publicado recentemente por Cechin e colaboradores [54] demonstrou que o tratamento com Exendin-4 *in vitro* reduz a produção de citocinas pró-inflamatórias em ilhotas humanas isoladas.

Além dos efeitos já vastamente investigados do GLP-1 na biologia da célula β, mais recentemente, esse hormônio também tem apresentado ações interessantes no tecido hepático. De fato, diferentes estudos evidenciaram a presença de GLP-1R na superfície de hepatócitos, sugerindo uma potencial ação do GLP-1 no fígado [55]. A estimulação do GLP-1R apresentou efeitos protetores em diferentes modelos de doenças hepáticas, tais como *nonalcoholic steatohepatitis* (NASH) e *nonalcoholic fatty liver disease* (NAFLD) [56, 57].

1.4 O mecanismo de indução da secreção insulínica pelo GLP-1 e sua possível relação com o controle bioenergético da célula β

O GLP-1 é uma incretina intestinal capaz de induzir fisiologicamente a secreção de insulina dependente de glicose a partir das células β pancreáticas [58]. Tal comunicação entre o trato gastrintestinal e o pâncreas endócrino foi primeiramente observada há mais de 50 anos, quando ficou demonstrado que a administração oral de glicose provocava um aumento significativamente maior nos níveis de insulina plasmática quando comparado com glicose administrada por via intravenosa. Esse

fenômeno foi denominado "efeito incretina", e é responsável por cerca de 50% a 70% da insulina total secretada após a ingestão oral de glicose [59, 60]. A primeira incretina a ser isolada foi a *glucose-dependent insulinotropic polypeptide* (GIP), que é secretada a partir das células K do intestino delgado superior, em resposta à ingestão de glicose e gordura. O GLP-1 foi identificado posteriormente e é produzido pelas células-L, localizadas principalmente no intestino delgado distal e no cólon [61].

A capacidade das incretinas de atuar como secretagogos e promover a secreção de insulina dependente de glicose tem sido amplamente investigada durante as últimas décadas [62]. No DM2, a secreção de GIP é normal, no entanto, a sua eficácia nas células β é muito reduzida, tornando a administração de GIP uma abordagem terapêutica ineficiente [63]. Por outro lado, a secreção de GLP-1 é reduzida, mas a sua ação é normal em pacientes com DM2 [64]. Assim, a reposição terapêutica dessa incretina é justificada e, de fato, tem sido verificada como eficiente na prática clínica [65]. Com o aumento da utilização de tais análogos para o tratamento do DM2, a elucidação dos mecanismos moleculares, através dos quais o GLP-1R transduz o sinal e promove a secreção de insulina, tornou-se fundamental. No entanto, tais mecanismos ainda não são totalmente caracterizados e o estado atual do conhecimento não pode explicar a plenitude das mudanças fisiológicas ocorridas nas células β em resposta à estimulação do GLP-1R.

Para compreender como o GLP-1 induz a liberação de insulina, é importante definir primeiro o mecanismo molecular básico que controla a secreção da mesma a partir das células β. A secreção de insulina é um processo complexo e altamente regulado [66]. Resumidamente, o *Glucose transporter 2* (GLUT2) medeia a entrada de glicose nas células β. A glicose é então fosforilada pela enzima glucoquinase e, após, catabolizada pela via da glicólise e fosforilação oxidativa mitocondrial, gerando

ATP. Isso causa um aumento na razão ATP/ADP, levando os canais de potássio dependentes de ATP a fechar e interrompendo o transporte de íons potássio através da membrana plasmática. Uma maior concentração de íons potássio no interior da célula causa a despolarização da membrana e, consequentemente, a ativação dos *voltage dependent calcium channels* (VDCCs). Isso provoca um rápido aumento das concentrações intracelulares de cálcio, resultando na exportação dos grânulos de insulina por exocitose. Assim, o metabolismo glicolítico e mitocondrial, por meio da geração de ATP, constitui o ponto central de controle da secreção insulínica. Mecanismos capazes de promover o metabolismo celular e a produção energética, pelo menos teoricamente, possuem o potencial para exacerbar a secreção de insulina.

A sinalização do receptor GLP-1R se inicia com a ligação do GLP-1 na membrana plasmática das células β. O GLP-1R é um receptor com domínio transmembrana sete passantes acoplado à proteína G. Após a ligação ao seu ligante, o GLP-1R ativa a proteína G trimérica, que, por sua vez, promove a ativação da adenilciclase ligada à membrana, levando à produção de cAMP [67]. A maioria, se não todos os efeitos conhecidos *downstream* à ativação do GLP-1R, em células β, são mediados por um ou por outro dos dois principais efetores do cAMP, a *protein kinase A* (PKA) e o c*AMP-regulated guanine nucleotide exchange factor* (cAMP GEF). Este também conhecido como *exchange protein activated by cAMP* (EPAC) [68].

Uma revisão recente de Meloni e colaboradores discute o conhecimento atual dos mecanismos pelos quais o GLP-1 sinaliza para a secreção de insulina dependente de glicose [69]. Vários mecanismos são sugeridos para mediar os efeitos do GLP-1 em células β após a geração de cAMP pela ação direta do receptor na ativação da adenilciclase. Estes, provavelmente atuam em conjunto para gerar o resultado final de aumento na secreção de insulina. Tais mecanismos incluem a inibição da abertura dos

canais de potássio dependentes de ATP, facilitando a abertura dos VDCCs. Adicionalmente, os efetores do cAMP também podem causar inibição da repolarização da membrana através do bloqueio dos *voltage-gated potassium channel* (Kv). Também ficou demonstrado que a ativação do GLP-1R facilita a liberação de cálcio induzida por cálcio (CICR) a partir de compartimentos de armazenamento citoplasmáticos, tais como o retículo endoplasmático [69].

Um estudo recente realizado por Sam Van de Velde e colaboradores demonstrou que cAMP gera duas ondas de ativação de expressão gênica em ilhotas primárias de rato [70]. A primeira ocorre apenas duas horas após a elevação do cAMP e é controlada pelo *cAMP response element-binding protein* (CREB). Já a segunda apenas se inicia após 16 horas e é controlada pelo *Hypoxia-inducible factor 1* (HIF-1). CREB regula a expressão de vários genes essenciais para a função das células β, incluindo o gene da insulina [71]. HIF-1 é um fator de transcrição que regula respostas de estresse celular, como a adaptação à hipóxia, mas também a angiogênese e o desenvolvimento fetal [72-74]. Ele também tem como alvo e induz a expressão de vários genes envolvidos na via glicolítica [75]. Em células β, o cAMP promove a tradução da subunidade alfa do HIF-1 (HIF-1α) em um mecanismo dependente da ativação da *mammalian Target of Rapamycin* (mTOR) [70]. Atualmente, acredita-se que o HIF-1α não apenas é essencial durante estresse, mas também para o metabolismo basal da célula β. De acordo com essa hipótese, o *knockout* condicional do HIF-1α somente em células β causou intolerância à glicose e secreção deficiente de insulina em camundongos [76].

2. JUSTIFICATIVA, HIPÓTESES E OBJETIVOS

2.1 Justificativa e hipótese partes 1 e 2

O estresse inflamatório produzido pela ME do doador causa danos teciduais irreversíveis em diversos tecidos e órgãos destinados a transplante. Os efeitos benéficos que a Exendin-4 exerce sobre ilhotas pancreáticas e fígado em vários modelos experimentais distintos sugerem que essa droga tenha o potencial de prevenir danos inflamatórios. Assim, nós hipotetizamos que, quando administrada a doadores em ME, a Exendin-4 pode amenizar o dano tecidual, melhorando parâmetros de qualidade desses tecidos. Se confirmada, essa hipótese poderá ser traduzida em uma estratégia inovadora para preservação da qualidade de tecidos doados, possivelmente, melhorando os desfechos de pacientes transplantados.

2.2 Objetivos partes 1 e 2

Determinou-se o efeito da administração de Exendin-4 a animais com ME em relação a diversos parâmetros de dano e qualidade teciduais, além de marcadores inflamatórios e expressão gênica de genes ligados a inflamação e estresse. Os resultados referentes à execução desses objetivos estão apresentados nas **partes 1 e 2** desta tese ("Exendin-4 protects rat islets against loss of viability and function induced by brain death"− publicado na revista *Molecular and Cellular Endocrinology,* em 2015 − e "Exendin-4 attenuates brain death-induced liver damage in the rat" − publicado na revista *Liver Transplantation,* em 2015).

2.3 Justificativa e hipótese parte 3

Considerando-se que o controle da secreção de insulina está intimamente ligado ao metabolismo de glicose na célula β, o que gera a base para o acoplamento metabólico "estímulo-secreção", nós sugerimos que o GLP-1 promove a secreção de insulina dependente de glicose por meio da modulação bioenergética, que inclui o aumento da taxa glicolítica de células β. Esse controle metabólico seria regulado pela via GLP-1R/mTOR/HIF-1α e consequente ativação da expressão gênica de genes codificadores de enzimas glicolíticas. Com esse estudo, buscou-se uma melhor compreensão dos mecanismos moleculares envolvidos na regulação da secreção de insulina pelo GLP-1.

2.4 Objetivos parte 3

Pretendeu-se realizar uma avaliação global dos efeitos da sinalização do GLP-1R na bioenergética das células β, bem como na expressão gênica de genes envolvidos na via glicolítica. Para isso, a linhagem de células β de rato, BRIN-BD11, e as ilhotas primárias isoladas de camundongos foram incubadas em várias condições experimentais na presença ou na ausência de Exendin-4. Estudos funcionais usando inibidores específicos de proteínas quinase e siRNAs dirigidos para HIF-1α foram realizados de forma a determinar com precisão as vias moleculares responsáveis pelo controle metabólico exercido pelo GLP-1. Os resultados desse estudo estão apresentados na **parte 3** desta tese ("GLP-1 receptor signaling promotes β-cell glucose metabolism *via* mTOR-dependent HIF-1α activation" − submetido para publicação na revista *Diabetes*).

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PARTE 1

Exendin-4 protects rat islets against loss of viability and function induced by brain death

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Exendin-4 protects rat islets against loss of viability and function induced by brain death

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Keywords: Brain death; islet transplantation; Exendin-4; inflammation, ER stress

***Abbreviations**:* Actb, β-actin; BD, brain death; Bip, Immunoglobulin heavy-chain binding protein; Ccl2, Chemokine (C-C motif) Ligand 2; Chop, C/EBP homologous protein; CREB, cAMP response element-binding protein; DAB, diaminobenzidine tetrahydrochloride; DAPI, 4′,6-diamidino-2-phenylindole, dilactate; eIF2α, Eukaryotic Initiation Factor 2; ER, endoplasmic reticulum; ERK1/2, Extracellular Signal-Regulated Kinases 1 and 2; Ex-4, Exendin-4; FDA, fluorescein diacetate; GLP1, Glucagon-Like Peptide-1; GSIS, glucose stimulated insulin secretion; HMGB1, High mobility group box 1; HRP, horseradish peroxidase; IBMIR, instant blood mediated inflammatory reaction; IEQ, islet equivalents; IL-1β, Interleukin-1 beta; IRE-1α, Inositol-Requiring Enzyme 1; KRB, Krebs-Ringer-Bicarbonate Buffer; MAPK, Mitogen-activated protein kinase; PI, propidium iodide; PKA, Protein kinase A; ROS, reactive oxygen species; Sod2, Superoxide Dismutase 2; Tf, Tissue factor; TNFα, Tumor Necrosis Factor; TRITC, tetramethylrhodamine; TUNEL, Terminal deoxynucleotidyl transferase dUTP nick end labeling; Ucp2, Uncoupling Protein 2; Xbp-1, X-box binding protein 1.

ABSTRACT

Islet quality loss after isolation from brain dead donors still hinders the implementation of human islet transplantation for treatment of type 1 diabetes. In this scenario, systemic inflammation elicited by donor brain death (BD) is among the main factors influencing islet viability and functional impairment. Exendin-4 is largely recognized to promote anti-inflammatory and cytoprotective effects on β-cells. Therefore, we hypothesized that administration of Exendin-4 to the BD-donors might improve islet survival and insulin secretory capabilities. Here, using a rat model of BD, we show that Exendin-4 administration to the brain dead islet donors increases both viability and glucose-stimulated insulin secretion. In this model, Exendin-4 treatment produced a significant decrease in *Interleukin-1 beta* gene expression in the pancreas. Furthermore, Exendin-4 treatment increased the expression of *Superoxide dismutase* and prevented BD-induced elevation in *Uncoupling protein 2* expression. Such observations were accompanied by a reduction in gene expression of two genes often associated with endoplasmic reticulum (ER) stress response in freshly isolated islets from treated animals, *C/EBP homologous protein* and *Immunoglobulin heavy-chain binding protein.* As ER stress response has been shown to be triggered by and to participate in cytokine-induced β-cell death, we suggest that Exendin-4 might exert its beneficial effects through alleviation of pancreatic inflammation and oxidative stress, which in turn could prevent islet ER stress and β-cell death. Our findings might unveil a novel strategy to preserve islet quality from BD donors. After testing in the human pancreatic islet transplantation setting, this approach might sum to the ongoing effort to achieve consistent and successful single-donor islet transplantation.

1. INTRODUCTION

Pancreatic islet transplantation has emerged as a promising treatment for type 1 diabetes mellitus (Ryan, et al. 2005; Shapiro, et al. 2000; Shapiro, et al. 2006). However, long term insulin independence only is achieved if a high mean islet mass (i.e., 10,000–14,000 islet equivalents per kilogram of recipient's body weight) is transplanted (Mineo, et al. 2009). Labile diabetic patients, who experience extreme oscillations in blood glucose levels, frequently leading to episodes of hypoglycemia, often benefit from grafts with a partial function. Improvement in glycemic control and hypoglycemia awareness are normally observed in patients transplanted with a sub-optimal islet mass (Leitao, et al. 2008). Yet, the ultimate goal of long-term insulin independence usually requires more than one organ donor. Therefore, most recipients receive islets from 2-3 donors, limiting the number of transplantations and, consequently, the number of patients benefiting from this therapy (Fiorina, et al. 2008). Owing to the above-mentioned reasons, developing new strategies to prevent islet quality loss during the isolation procedure is of high interest.

Brain death (BD) exerts an important negative impact on deceased-donor organ quality (Pratschke, et al. 1999). This impact has been demonstrated in various organs such as liver, kidney and heart (Pratschke, et al. 2001; Shivalkar, et al. 1993; Weiss, et al. 2007). In rat pancreatic islets, BD was shown to reduce both viability and function after isolation, probably through an increase in pro-inflammatory cytokines (Contreras, et al. 2003). Furthermore, our group has previously shown that BD induces inflammation in human pancreatic tissue (Rech, et al. 2014). Inflammation is well known to mediate β-cell death and participates in the pathogenesis of both type 1 and type 2 diabetes (Eizirik and Mandrup-Poulsen 2001). Accumulating evidence also

points to a role for inflammation in triggering reactive oxygen species (ROS) production and oxidative damage to β-cells (Barbu, et al. 2002; Chen, et al. 2005; Tabatabaie, et al. 2003).

Glucagon-like peptide-1 (GLP1) analogs have been approved as a therapy for type 2 diabetes, since they increase insulin secretion, decrease glucagon production and delay gastric empting (Drucker and Nauck 2006). Anti-inflammatory and cytoprotective properties of GLP1 analogs, acting directly on β-cells, have been extensively supported by many studies. Such analogs have been shown to prevent β-cell apoptosis triggered by different stimuli such as lipotoxicity, pro-inflammatory cytokines, glucocorticoids and streptozotocin (Ferdaoussi, et al. 2008; Li, et al. 2003; Natalicchio, et al. 2013; Ranta, et al. 2006). Many molecular mechanisms and intracellular signal transduction pathways are believed to be involved in the protective actions of GLP1 analogs. Upon ligand binding, GLP1 receptor activates adenylyl cyclase, increasing intracellular cAMP levels, which in turn activates Protein kinase A (PKA). Regarding metabolic actions of GLP1 analogs on β-cells, PKA activation and subsequent phosphorylation of its downstream targets is the primary pathway to potentiate glucose-dependent insulin secretion (Kaihara, et al. 2013). However, cAMP signaling also activates targets involved in the cytoprotective actions of GLP1 analogs (Jhala, et al. 2003). In fact, several studies reported anti-inflammatory actions of cAMP signaling on β-cells and other cell types (Pugazhenthi, et al. 2010). Remarkably, the transcription factor cAMP response element-binding protein (CREB) is activated upon cAMP signaling and promotes the expression of numerous genes associated with cell survival during stress conditions. Furthermore, Exendin-4 has also been shown to induce activation of Extracellular Signal-Regulated Kinases 1 and 2 (ERK1/2), which play important roles in cell survival, proliferation, insulin

secretion and protection against apoptosis (Cechin, et al. 2012). Another cellular process implicated in β-cell death is the Endoplasmic Reticulum (ER) stress response (Huang, et al. 2007; Kharroubi, et al. 2004; Oyadomari, et al. 2001). Interestingly, GLP1 receptor agonists also protect β-cells from cytotoxicity promoted by ER stress inducing agents (Cunha, et al. 2009; Tsunekawa, et al. 2007).

We hypothesized that the administration of the GLP1 analog Exendin-4 to the brain dead donor, due to its cytoprotective properties, might improve islet quality parameters, important to improve transplantation success rates. Here, we show that Exendin-4 administration, following BD, increases isolated islets viability and function, which is accompanied by attenuation of pancreatic gene expression of *Interleukin-1 beta (IL-1β)* and stress related genes.

2. MATERIALS AND METHODS

2.1 Animals

Male Wistar rats, fed standard laboratory diet ad libitum, weighting 300-350 g, were used throughout the study. All animals were kept in the animal facility of the Hospital de Clínicas de Porto Alegre (Porto Alegre, Brazil) and were cared according to the guidelines for use and care of laboratory animals (National Research Council (U.S.). Committee for the Update of the Guide for the Care and Use of Laboratory Animals., et al. 2011). The study was approved by the local ethics committee for the use of animals in research.

2.2 Experimental design

Animals were randomized in three experimental groups: Control, no central

nervous system injury; BD, brain death induced experimentally; and BD+Ex-4, brain death induced experimentally, followed by immediate intraperitoneal administration of Exendin-4 (Exenatide; Eli Lilly, Indianapolis, IN, USA) 5μg/Kg of body weight. A schematic diagram outlining the experimental procedures was included in (Fig. 1*A*). First, animals were anesthetized by a mixture of 2% isoflurane (Biochimico, Itatiaia, Brazil) in oxygen using a precision vaporizer (Takaoka, model 1415, Sao Paulo, Brazil). Then, they were intubated using a plastic cannula from a 16-gauge catheter (Abbocath®; Abbott Laboratories, Abbott Park, IL, USA) and mechanic ventilated using a volumetric ventilator (Harvard Rodent Ventilator, model 683; Harvard Apparatus Co., Millis, MA, USA) with a tidal volume of 2.5 mL and frequency of 85 strokes per minute. Arterial blood pressure was monitored continuously through cannulation of the left femoral artery, attached to a Biopac MP100 data acquisition system (Biopac Systems Inc. Goleta, CA, USA). Brain death was induced using a modified version of the method previously published by Takada et al. (Takada, et al. 1998). Briefly, a hole was drilled at the left front lateral region of the animal's skull to permit the passage of a No. 4 Fogarty catheter (Fogarty Arterial Embolectomy Catheter, 4F; Edwards Lifesciences, Irvine, CA, USA) without direct cerebral damage. The balloon was then rapidly inflated with 0.5 mL of saline for one minute. Animals in the control (sham-operated) group had the catheter inserted but not inflated. Maximally dilated pupils, apnea and absence of palpebral reflexes confirmed the BD condition. Body temperature was preserved at 37^0C by means of a heated dorsal table. Animals were maintained for six additional hours prior to either pancreas biopsy or collagenase perfusion for islet isolation.

2.3 Islet isolation and dispersion

Islet isolation has been performed similarly to the protocol suggested by Carter et al. (Carter, et al. 2009). After six hours of mechanical ventilation, the bile duct was cannulated to allow pancreatic perfusion with 10 mL of cold Hank's balanced salt solution (HBSS, Sigma-Aldrich) containing 0.5 mg/mL of Collagenase P (Roche Diagnostics, Mannheim, Germany). The perfused pancreas was removed and digested for 15 minutes at 37^0C. Digestion was stopped by the addition of RPMI 1640 media (11 mmol/L glucose), supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 0.1 mg/ml streptomycin (Gibco Life Technologies, Gaithersburg, MD, USA), and 25 mmol/l HEPES. Islet purification was performed in Histopaque (Sigma-Aldrich) density gradient using 1.119, 1.100, 1.077 g/mL density layers. An aliquot of purified islets was counted as islet equivalent (IEQ) numbers under a scaled microscope. One IEQ was the islet tissue mass equivalent to a spherical islet of 150 μm in diameter. After isolation, islets were immediately washed with HBSS and stored at -80^0C for protein and RNA extraction. When dispersion was needed, islets were digested with 0.125% Trypsin-EDTA (Gibco Life Technologies, Gaithersburg, MD, USA) for five minutes at 37^0C. Digestion was stopped by addition of supplemented RPMI 1640 and the resulting islet single-cell suspension immediately evaluated for viability or nuclear chromatin staining. For glucose stimulated insulin secretion assays, islets were rested for 3 hours in supplemented RPMI 1640 at 37^0C in a humidified atmosphere of 95% air and 5% CO_2 prior to static incubations.

2.4 Islet viability

Upon fluorescein diacetate (FDA) and propidium iodide (PI) staining, living cells actively convert the non-fluorescent FDA into the green fluorescent compound

fluorescein, while dead cells show red fluorescence in their nuclei due to PI entrance through the permeabilized membrane. In order to avoid the limitation of this method to evaluate islet viability in intact islets (Boyd, et al. 2008), we used dispersed islet cells to perform viability assessment. For this, FDA-PI staining was used to distinguish dead from viable single cells. Freshly dispersed islet cells were stained with 5 μg/mL FDA and 5 μg/mL PI (Sigma-Aldrich) and immediately analyzed under an Axio Imager A2 fluorescent microscope (Carl Zeiss Microscopy GmbH, Jena, Germany). For each animal 1000 cells were counted and the percentages of viable and dead cells estimated. Images were acquired using a digital camera (Zeiss - Axio Cam HRc®) and the Axio Vision software (Carl Zeiss Microscopy GmbH, Jena, Germany).

2.5 Glucose stimulated insulin secretion

Glucose stimulated insulin secretion (GSIS) was performed on freshly isolated islets after resting them for 3 hours in supplemented RPMI 1640 at 37^0C in a humidified atmosphere of 95% air and 5% CO_2. Briefly, 15 islets transferred into 1 mL of Krebs-Ringer-Bicarbonate Buffer (KRB, 137 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, 0.25% BSA, 2.8 mM glucose) in triplicates and incubated for 30 minutes at 37^0C in a humidified atmosphere of 95% air and 5% CO_2. The media was removed and replaced by another 1 mL of fresh KRB, followed by one-hour incubation at the same conditions (basal glucose stimulation). This time, media was removed, stored for insulin measurement and replaced by 1 mL of KRB containing 28 mM glucose (high glucose stimulation). After additional one-hour incubation, media was collected and stored for insulin measurement. Islet DNA was extracted by boiling the samples in 100 μL of TE buffer

(10 mM Tris-HCl, 1 mM EDTA, pH 8.0) for 20 minutes, and total DNA was quantified using Quanti-iT PicoGreen (Invitrogen Life Technologies, Gaithersburg, MD, USA). Secreted insulin in the media was assessed by Rat Insulin ELISA kit (Millipore, Billerica, MA, USA) according to manufacturer instructions. Secreted insulin was normalized by total DNA and expressed as picograms of insulin / nanograms of DNA / hour. For stimulation index (SI) assessment, the ratio between high to basal glucose stimulation was calculated.

2.6 Quantitative RT-PCR

RNA was extracted from islets and pancreatic tissue using RNeasy Mini Kit (Qiagen, Chatsworth, CA) and was reverse-transcribed using SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen Life Technologies, Gaithersburg, MD, USA) according to manufacturer's protocols. Quantitative PCR was performed by monitoring the increase in fluorescence of Fast SYBR® Green Master Mix (Invitrogen Life Technologies, Gaithersburg, MD, USA) (Higuchi, et al. 1993). Rat specific primer sequences for the genes *IL-1β*, *Tumor necrosis factor* (*TNF-α*), *Chemokine (C-C motif) ligand 2* (*Ccl2*), *Tissue factor* (*Tf*), *C/EBP homologous protein* (*Chop*), *Immunoglobulin heavy-chain binding protein* (*Bip*), *Superoxide dismutase 2* (*Sod2*), *Uncoupling protein 2* (*Ucp2*), and *β-actin* (*Actb*) were designed using Primer Express 3.0 software (Life Technologies, Gaithersburg, MD, USA) and are represented in Table 1. All the reactions were performed in a Viia^{TM} 7 real time PCR system (Life Technologies, Gaithersburg, MD, USA). Relative mRNA expression was calculated through the standard curve method (Wong and Medrano 2005) using β-actin as the reference gene. Quantitative RT-PCR specificity was determined using melting curve analyses and all primers generated amplicons that

produced a single sharp peak during the analyses.

2.7 Immunoblot

Protein extracts from freshly isolated islets were resolved on 12% polyacrylamide gels, transferred to Immobilon®-P^{SQ} membranes (Millipore, Billerica, MA, USA), and incubated with monoclonal antibodies to Active Caspase-3, total ERK1/2, phosphoERK1/2 (Thr202/Tyr204) (Cell Signaling Technology, Beverly, MA, USA) and α-Tubulin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), Secondary antibodies consisted of horseradish peroxidase conjugated goat anti-mouse or anti-rabbit antibodies (Millipore, Billerica, MA, USA). Detection was performed using Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA, USA), and images were acquired in an ImageQuant LAS 500 (GE Healthcare, Piscataway, NJ, USA) digital imaging system and band densitometry analysis was performed using ImageJ 1.47v. In order to have a positive control for Caspase-3 activation, islets were cultured for 48 hours in standard culture conditions in the presence or absence of a cytokine mix containing the recombinant human cytokines IL-1β, TNF-α and IFNγ at 50, 1000 and 100 U/mL respectively (Gibco Life Technologies, Gaithersburg, MD, USA).

2.8 Immunohistochemistry

Pancreata were removed, fixed in 10% phosphate buffered formalin, embedded in paraffin, sectioned (4 μm thick), and immunostained after deparaffinization and rehydration. For Active Caspase-3 staining slides were incubated with primary antibody (Cell Signaling Technology, Beverly, MA, USA) and then with a biotinylated secondary antibody, streptavidin-horseradish peroxidase

conjugate, and diaminobenzidine tetrahydrochloride (DAB) (Dako Cytomation, Inc., Carpinteria, CA, USA). Quantification was performed by digital image analyses using Image Pro Plus 4.5 software (Media Cybernetics, Bethesda, MD, USA). Images were acquired using a Cool Snap-Pro CS (Media Cybernetics, Bethesda, MD, USA) camera attached to a Zeiss microscope (model AXIOSKOP-40; Carl Zeiss Microscopy GmbH, Jena, Germany). Then, islets were cropped from the original images in order to consider only staining within the islets. The area of each islet was calculated and recorded using ImageJ 1.47v (National Institutes of Health, USA). Two independent blinded investigators (R.C. and N.E.L) analyzed the intensity of brown-colored immunostaining in pixels, and a Pearson's correlation of r=0.83 was obtained between the two observers. The mean number of pixels identified by the two investigators normalized by the islet area was used to quantify Caspase-3 activation in 10 islets from each animal.

2.9 Immunofluorescence and terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) assay

Pancreata were removed, fixed in 10% phosphate buffered formalin, embedded in paraffin, sectioned (4 μm thick), and immunostained after deparaffinization and rehydration. For insulin staining, slides were incubated with anti-insulin primary antibody (Dako Cytomation, Inc., Carpinteria, CA, USA) and then with a tetramethylrhodamine (TRITC) conjugated anti-guinea pig secondary antibody (Sigma-Aldrich). Finally, slides were mounted with 4′,6-diamidino-2-phenylindole, dilactate (DAPI) containing mounting medium (Sigma-Aldrich) for nuclei staining. For TUNEL assay, following deparaffinization and rehydration, slides were assayed with In Situ Cell Death Detection Kit (Roche Diagnostics, Mannheim,

Germany), according to the manufacturer's protocol. For positive controls, slides were pre-treated with one unit of DNAse I for 15 minutes at 37^0C, in order to generate double stranded DNA breaks. Both immunofluorescence and *in situ* TUNEL assays were analyzed under an Axio Imager A2 fluorescent microscope (Carl Zeiss Microscopy GmbH, Jena, Germany). Images were acquired using a digital camera (Zeiss - Axio Cam HRc®) and the Axio Vision software (Carl Zeiss Microscopy GmbH, Jena, Germany).

2.10 Nuclear chromatin staining

Freshly dispersed islet cells were stained with 10 μg/ml Hoechst 33342 (Sigma-Aldrich) for 10 minutes at 37^0C, as described in (Riachy, et al. 2002). To quantify apoptosis 1000 nuclei were counted per animal and apoptotic cells were identified by the presence of condensed or fragmented nuclei. Counting and image acquisition were performed under an Axio Imager A2 fluorescent microscope, attached to a digital camera with the aid of the Axio Vision software (Carl Zeiss Microscopy GmbH, Jena, Germany).

2.11 Caspase-Glo 3/7 assay

After isolation, islets were immediately frozen and protein extracts obtained and quantified. Then, 10 μg of total protein lysates were assayed for Caspase 3 and 7 activities by Caspase-Glo 3/7 assay (Promega Corporation, Madison, WI, USA) according to manufacturer instructions.

2.12 Statistical analysis

Variables are described as mean ± S.E.M. Variables with non-normal distribution were log-transformed. Differences across groups were tested using ANOVA with a Tukey post hoc test. Statistical significance was set at $P < 0.05$ (two-tailed). All analyses were performed using SPSS 18 (IBM, Armonk, NY, USA).

3. RESULTS

3.1 Exendin-4 prevents loss of islet cell viability induced by brain death

Islet viability >80% is among the product release criteria for human islet transplantation (Davide Mineo and Rodolfo Alejandro 2010). Moreover, BD has previously been shown to promote loss of islet cell viability (Contreras et al. 2003). In order to evaluate the effect of Exendin-4 administration on single cell viability, we assayed dispersed islet cells by FDA-PI staining. Islets originated from the BD group showed a decrease in viable cells (proportion of FDA positive cells) as compared with control islets from sham-operated animals. Administration of Exendin-4 following the induction of BD protected pancreatic islets against the viability loss observed in the BD group (control 86.6% ± 4.85%; BD 59.8% ± 15.46%; BD+Ex-4 91.6% ± 0.57%, P< 0.05) (Fig. 1*B*). In accordance, on BD group-derived islets, we observed a significant 3-fold increase in the proportion of dead cells (positively stained by PI). This observation was also reversed by Exendin-4 treatment (Fig. 1*C*). Figure 1*D* depicts representative fluorescence micrographs from the three experimental groups.

It has been previously shown that BD impacts islet recovery after purification (Contreras et al. 2003). We quantified post purification islet recovery from our three

experimental groups, although no statistic significant differences were observed (control 910 ± 266; BD 865 ± 378; BD+Ex-4 728 ± 391, IEQs/pancreas, P= 0.81).

3.2 Brain death impairs glucose-stimulated insulin secretion and Exendin-4 treatment minimizes such effect

Functionality of pancreatic islets, as estimated by their ability to secrete insulin in response to glucose stimulation, has also been shown to be impaired after BD (Contreras et al. 2003). We investigated the ability of Exendin-4 to protect islets against BD-induced dysfunction. For this purpose, insulin secretion was evaluated by static incubation experiments at two glucose concentrations: basal (2.8 mM glucose) and stimulated (28 mM glucose). At basal glucose stimulation conditions the insulin secretion did not differ statistically among groups. However, when islets were stimulated at 28 mM glucose, insulin secretion from the BD group was reduced as compared with both control and Exendin-4 treated groups (control 0.88 ± 0.1; BD 0.56 ± 0.07; BD+Ex-4 0.83 ± 0.07, pg of insulin / ng of DNA / hour, P< 0.01) (Fig. 2*A*). The stimulation index, which represents the insulin secretion ratio (stimulated/basal), also differed among groups. Again, islets derived from the BD group showed insulin secretion impairment when compared with control group islets, and Exendin-4 administration was able to protect against such damage caused by BD (control 6.2 ± 0.41; BD 5.1 ± 0.6; BD+Ex-4 6.1 ± 0.35, P< 0.05) (Fig. 2*B*).

The activation status of ERK1/2, the most downstream member of the Mitogen-activated protein kinase (MAPK) pathway, is essential for insulin gene expression and secretion in response to glucose (Wijesekara, et al. 2010). In addition, several reports have demonstrated the ability of GLP1 analogs to promote ERK1/2 phosphorylation/activation in β-cells (Egan, et al. 2003). Thus, we assessed the

phosphorylation status of ERK1/2 in islets isolated from the three experimental groups. Donor treatment with Exendin-4 significantly increased the phosphorylation states of both ERK1 and ERK2 (Fig. 2*C* and 2*E*).

3.3 Brain death increases mRNA expression of pro-inflammatory cytokines in the pancreas and Exendin-4 treatment partially prevents it

Brain death has been shown to induce the gene expression of pro-inflammatory cytokines in different organs, including the pancreas (Contreras et al. 2003; Takada et al. 1998). We hypothesized that an inflammatory pancreatic environment could be the physiological mechanism leading to the observed BD-induced impairment in β-cell function and viability. Thus, we assessed pancreatic mRNA expression of *IL-1β* and *TNF-α*, two cytokines widely associated with β-cell death. Pancreata from BD animals exhibited significantly increased *IL-1β* and *TNF-α* gene expressions as compared with control animals (Fig. 3*A* and 3*B*, respectively). Exendin-4 treatment completely blocked *IL-1β* expression raise, whereas produced no significant effect on *TNF-α* expression. We also assessed the expression of *Ccl2* and *Tf*, two genes that have their expression associated with instant blood mediated inflammatory reaction (IBMIR) in the transplantation setting (Saito, et al. 2010). No significant differences in pancreatic mRNA expression were found among the groups for these two genes (*Ccl2* - control 1.00 ± 0.57; BD 1.74 ± 1.00; BD+Ex-4 1.38 ± 0.87, P=0.90) and (*Tf*; control 1.00 ± 0.22; BD 1.35 ± 0.47; BD+Ex-4 1.15 ± 0.66, P=0.66). Gene expression of *IL-1β*, *TNF-α*, *Ccl2* and *Tf* were also evaluated in freshly isolated islets and no significant differences were observed among groups (data not shown).

3.4 Exendin-4 promotes pancreatic expression of *Sod2* and prevents BD-induced increase in *Ucp2*

Oxidative stress is believed to play a role in BD-induced organ damage (Morariu, et al. 2008). In addition, Exendin-4 has recently been shown to have interesting antioxidant properties (Padmasekar, et al. 2013). This prompted us to investigate pancreatic expression of two oxidative stress related genes, *Sod2* and *Ucp2*, in our experimental model. Both genes were elevated in BD as compared to control animals (Fig. 4*A* and 4*B*). Exendin-4 treatment further up-regulated gene expression of the mitochondrial antioxidant enzyme *Sod2* (Fig. 4*A*). In contrast, *Ucp2*, which was significantly increased in BD animals, returned to control levels in the Exendin-4 treated group (Fig. 4*B*).

3.5 *Chop* and *Bip* gene expression is augmented on islets from brain dead animals and Exendin-4 treatment protects against it

Several studies have evidenced that ER stress may contribute to cytokine mediated β-cell death [reviewed in (Eizirik, et al. 2008)]. Furthermore, Exendin-4 has been shown to protect β-cells from ER stress (Cunha et al. 2009; Tsunekawa et al. 2007). This prompted us to investigate the association between BD and islet expression of *Chop* and *Bip*, two genes known to be up-regulated and widely used as markers of ER stress, as well as the effect of Exendin-4 on this scenario. Here, we show that the mRNA expressions of both genes are elevated on islets originated from brain dead animals (Fig. 5*A* and 5*B*). Strikingly, treating animals with Exendin-4 protected islets against such elevation on ER stress-related genes.

3.6 Brain death-induced loss of viability is not elicited by classic apoptosis

In an attempt to evaluate to which extend BD promotes islet apoptosis, we performed Immunoblot to active-Caspase-3 on freshly isolated islets from three animals of each experimental group. Surprisingly, Caspase-3 activity was below detection limit in all animals (Fig. 6*A*). In order to have a positive control for the experimental system, we treated islets with a cytokine mix as described in *Research Design and Methods*. Figure 5*B* shows clear activation of Caspase-3 upon exposure to cytokines, suggesting that in our BD model there is no Caspase-3 activation and, consequently, no evidence of classic apoptosis. To confirm this finding, we performed immunohistochemistry for active-Caspase-3 on pancreas biopsies from six animals of each experimental group. No statistical significant difference was found among groups using this method (Fig. 7*A* and 7*B*). We also counted the appearance of condensed nuclei on dispersed islet cells from all groups. This has been done by analyzing Hoechst 33342 stained cells under fluorescence microscope. Here, again, no significant differences were observed among groups (Fig. 7*C* and 7*D*). Contreras et al., using a different rat model of BD, reported that TUNEL staining is increased in the pancreatic tissue of BD donors (Contreras et al. 2003). In our model no increase in TUNEL staining was observed in response to BD (Fig. 7*E*). In order to determine the overall impact of BD in terms of islet architecture and insulin content, we performed insulin immunofluorescence in tissue sections from the three experimental groups. Danobeitia et al., have recently shown that BD promotes loss of insulin staining and morphological integrity of islets in a non-human primate model of BD (Danobeitia, et al. 2014). In our model, however, such alterations are not apparent prior to islet isolation (Fig. 7*F*). At last, islet protein extracts from control and BD animals were assayed for Caspase-3 and Caspase-7 activities using the Caspase-Glo 3/7 assay (Fig. 7G). No significant differences were observed between the groups. Altogether, our

data suggest that the cell death observed following 6 hours of BD is not classical apoptosis.

4. DISCUSSION

In our experimental model, BD induced loss of islet viability and function. These outcomes were associated with an increase in mRNA gene expression of pro-inflammatory cytokines *IL-1β* and *TNF-α*, as well as the oxidative stress-related genes *Sod2* and *Ucp2* in pancreatic tissue. Additionally, ER stress response related genes were augmented in islets isolated from BD donors. Interestingly, Exedin-4 treatment after the BD induction was able to improve islet function and viability, which was accompanied by a decrease in *IL-1β*, *Chop* and *Bip*. Furthermore, Exedin-4 treatment further enhanced the expression of *Sod2,* a mitochondrial antioxidant enzyme, and prevented BD-induced increase in *Ucp2*.

The Edmonton protocol, proposed by Shapiro et al. (Ryan et al. 2005; Shapiro et al. 2000; Shapiro et al. 2006), evidenced that human islet transplantation is a feasible alternative therapy for type 1 diabetes. However, the scarcity of available islets for transplantation restricts the adoption of this therapeutic alternative in the routine clinical practice. Although a few centers have reported success in transplanting recipients with islets from a single-donor (Hering, et al. 2005; Koh, et al. 2010), the general need for multiple donors still impends the advance of islet transplantation as a therapy option. Many approaches have been developed to avoid islet mass loss during isolation, culture, transplantation and post-transplantation stages (Shapiro 2011). Nonetheless, no single intervention has emerged as a sole method effectively enhancing single-donor islet transplantation success.

There is strong evidence suggesting an involvement of donor's BD in organ quality loss (Contreras et al. 2003; Pratschke et al. 1999; Pratschke et al. 2001; Rech et al. 2014; Shivalkar et al. 1993; Weiss et al. 2007). Use of living-donor pancreas for islet transplantation demonstrated that insulin independency was attained with as little as a half pancreas islet preparation (Matsumoto, et al. 2005). This represents an indirect piece of evidence suggesting that the donor's BD is, indeed, highly associated with the need for multiple donors to achieve insulin independency. However, living-donor islet transplantation is a therapy of exception because it might be associated with significant donor morbidity. In this sense, strategies minimizing the deleterious effects of BD have the potential to improve clinical islet transplantation outcomes.

Early organ donor management strategies have been used in transplantation centers and are associated with increased organ procurement (Rech, et al. 2013). In general, protocols concentrate on hemodynamic and hormonal control; however no approach focusing on specific pancreatic protection has been clinically evaluated so far. One study, using a rat gradual onset BD model, demonstrated that 17β-estradiol administration could improve islet quality (Eckhoff, et al. 2004), although it still awaits clinical investigation. Recently, a study by Danobeitia et al. demonstrated that pre-treatement with IL-1 receptor antagonist attenuated inflammation and improved islet function in a non-human primate BD model (Danobeitia et al. 2014). This study, along with ours, represents an important proof of concept for the hypothesis that BD donor treatment with anti-inflammatory and β-cell protective agents may improve post isolation islet function. Exendin-4 and Liraglutide, another GLP1 analog, have been shown to benefit islet transplantation outcomes when added to islet culture and/or administered to the recipients (Faradji, et al. 2009; Merani, et al. 2008; Toso, et al. 2010). Based on our findings, Exendin-4 has the potential to improve islet

transplantation outcomes when administered to the organ donor prior to pancreas procurement. With this strategy, islet damage could be prevented and probably a larger mass of islets would be available for transplantation. It is possible that benefits in terms of islet viability and function observed in this study may not be translated into long term graft survival *in vivo.* Thus, future research evaluating transplantation outcomes both in animal models and in a clinical setting must be done before the suggested donor management strategy can be adopted in the clinical practice.

In accordance with our gene expression analyses, we hypothesize that the cytoprotective effects of Exendin-4 observed here operate, at least in part, through a mechanism involving attenuation of pancreatic inflammation and oxidative stress. However, one cannot rule out that BD could affect vascular stability and expose pancreatic islets to hypoxic conditions. In this scenario, Exendin-4 could minimize islet cell death and by this reduce macrophage infiltration and, consequently, inflammation. Thus, data presented here cannot distinguish between a model in which inflammation is directly caused by BD or inflammation is a secondary effect caused by infiltrating macrophages clearing dying hypoxic cells. Figure 3*A* shows an important increase in *IL-1β* expression in the pancreas upon BD. Such elevation in *IL-1β* expression was completely abrogated in animals receiving Exendin-4. Our data also show that expression of another pro-inflammatory cytokine, *TNF-α*, is increased as a consequence of BD. In contrast to what has been found for *IL-1β*, Exendin-4 treatment had no effect on *TNF-α* expression. *In vitro* studies revealed that *TNF-α* has less pronounced β-cell death promoting properties than *IL-1β* (Ortis, et al. 2012). In our experimental setting, *TNF-α* seems to play no important role in the Exendin-4's islet protective mechanism. Future studies are necessary to determine why Exendin-4 treatment affected *IL-1β*, but has not changed *TNF-α* expression. It has been shown

that BD promotes intra-islet infiltration of macrophages (Danobeitia et al. 2014) and it might be that Exendin-4 treatment affects infiltration of specific immune cell populations. In agreement with this idea, Exendin-4 has previously been shown to diminish monocyte infiltration and adhesion molecules expression in a mouse model of atherosclerosis (Arakawa, et al. 2010). Thus, future research could investigate whether Exendin-4 affects the dynamics of immune cells infiltration in the pancreatic tissue of BD-donors.

IL-1β promotes β-cell death in a variety of experimental systems, so the sole decrease in its expression in the pancreatic environment could exert a positive impact on islet survival. This cytokine can activate various intracellular signaling pathways involved in β-cell death (Eizirik and Mandrup-Poulsen 2001). One mechanism more recently demonstrated to take part in *IL-1β*-induced β-cell death is the ER stress response (Cardozo, et al. 2005). Therefore, we decided to evaluate both, the pancreatic milieu, to which the islets are exposed, and a more downstream event, intrinsic to the islets, which is known to occur upon cytokine signaling, the ER stress response. It is noteworthy to mention that Exendin-4 has previously been shown to protect β-cells from ER stress (Cunha et al. 2009; Tsunekawa et al. 2007). In this study, we show for the first time that islet expression of *Chop* and *Bip,* two important components of the ER stress response, increases upon BD. More importantly, this increase is prevented by Exendin-4 treatment (Fig. 5A and 5B). *Chop* and *Bip* are commonly used in the literature as markers of ER stress; however, their expression could also be increased by other types of cellular stresses that can promote phosphorylation of the subunit alpha of *Eukaryotic Initiation Factor 2* (*eIF2α*), such as amino acid deprivation, presence of double-stranded RNA and heavy metals (Wek, et al. 2006). Therefore, they are not specific indicators of ER stress. In the particular

case of β-cells, previous studies have already demonstrated that *IL-1β* alone can promote Inositol-Requiring Enzyme 1 (*IRE-1α*) mediated *X-box binding protein 1* (*Xbp-1*) alternative splicing, an event specifically activated during ER stress response (Cardozo et al. 2005). Considering that our model produced a significant increase in pancreatic *IL-1β* expression, we suggest that Chop and BiP increase following BD could be an evidence of ER stress response activation in isolated islet cells.

Our data also point to a role for BD in triggering pancreatic oxidative stress. Many lines of evidence suggest that ROS production in response to a variety of stimuli, including pro-inflammatory cytokines, promotes β-cell death and GSIS impairment (Tabatabaie et al. 2003). We aimed at investigating the expression of two antioxidant genes *Sod2* and *Ucp2*, known to be upregulated in oxidative stress conditions (Pi, et al. 2010). *Sod2* is a mitochondrial manganese superoxide dismutase with an important role in protection against oxidative damage, and it is induced by pro-inflammatory cytokines (Darville, et al. 2000). *Ucp2* controls mitochondrial-derived ROS production. In addition, numerous studies have demonstrated a negative link between *Ucp2* expression and β-cell function (Robson-Doucette, et al. 2011). The two genes were augmented in the pancreas of BD animals (Fig.4), indicating a compensatory mechanism to counterbalance the increase in oxidative stress due to BD. Interestingly, Exendin-4 treatment further exacerbated *Sod2* expression, which was accompanied by a reduction in *Ucp2*. Our data is consistent with a previous study, which evidenced that increased superoxide levels induce *Ucp2* expression, and that such activation promotes β-cell dysfunction (Krauss, et al. 2003). Higher levels of *Sod2* observed in the Exendin-4 treated group supposedly potentiated superoxide clearance, presumably preventing activation of *Ucp2* in the BD group.

Our findings raise a debate regarding the type of cell death triggered by BD on islet cells. The study of Contreras et al. (Contreras et al. 2003) suggested that BD induces apoptotic cell death of the islet cells. Two pieces of evidence were presented to support such supposition. TUNEL assay and quantification of DNA fragmentation by ELISA showed an increase in islet cell apoptosis induced by BD. This conclusion is based on the same assumption that DNA fragmentation is a hallmark of apoptotic cell death. However, DNA fragmentation also occurs in necrotic cell death (Dong, et al. 1997) and TUNEL labeling of dead cells does not specifically indicate the underlying mechanism of death (Grasl-Kraupp, et al. 1995). We were not able to reproduce TUNEL assay results presented by Contreras et al. (Fig. 7*E*). Today, the gold standard marker of apoptotic cell death is activation of Caspase-3, which is involved in the execution phase of both intrinsic and extrinsic pathways of apoptosis. Using Caspase-3 activation assessment, both *in situ* by immunohistochemistry and in isolated islets by Immunoblot and Caspase-Glo assay, we were not able to detect any significant difference between BD and control animals (Figs. 6, 7*A* and 7*G*). Quantification of condensed nuclei, another hallmark of apoptotic cell death, showed no difference between the groups either, corroborating the other findings of this study (Fig. 7*B*). Recently, careful dissection of the biochemical mechanisms involved in β-cell death induced mainly by *IL-1β* has suggested that the primary type of death is not classical apoptosis. It does not involve Caspase-3 activation, at least in the initial six hours of exposure (Collier, et al. 2011; Collier, et al. 2006), and causes release of *High mobility group box 1* (HMGB1) (Steer, et al. 2006), a protein selectively released by cells undergoing necrosis. Our data support the hypothesis that the type of cell death that β-cells undergo after six hours of BD plus the isolation stress is necrosis and not classical apoptosis. It is noteworthy to mention that after long-term

exposure (e.g. 48h) cytokines can effectively induce Caspase-3 activation (Fig. 6*B*). We showed that BD causes a 3-fold increase in the number of PI stained cells with intact nuclei (Fig. 1*B*). PI only enters cells with compromised membrane integrity, a hallmark of either necrotic cell death or late-apoptotic cells. Microscopic examination can easily discriminate necrotic from late-apoptotic cells. Necrotic cells display intact nuclei, whereas late-apoptotic cells present fragmented nuclei. In our analyses, the number of cells presenting PI stained fragmented nuclei was negligible. Moreover, the absence of statistically significant differences in Caspase-3 activation indicates that the loss of cell viability observed in the BD group probably does not occur through apoptotic cell death.

Altogether, our findings demonstrate the potential of Exendin-4 as a protective agent for pancreatic islets when administered to the deceased organ donor prior to organ procurement. After thorough evaluation in a clinical trial, incorporation of this strategy in the clinical setting might add to the ongoing effort to attain consistent and successful single-donor islet transplantation for the treatment of type 1 diabetic patients.

5. Declaration of Interest

The authors declare no potential conflicts of interest relevant to this article.

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7. Author Contribution Statement

R.C. designed the study, researched data, and wrote the manuscript. N.E.L., A.L.D. F.S.O and L.A.B. researched data. L.H.C contributed to discussion and reviewed the manuscript. A.C.B. and C.B.L. designed the study and contributed to discussion. D.C. designed the study and reviewed/edited the manuscript. R.C. and D.C. are joint guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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9. Figure Legends

Figure 1. Islet cell viability loss induced by BD is prevented by Exendin-4 treatment. *A*, schematic diagram outlining the experimental procedures detailed in *Research Design and Methods*. Islet single cell viability was assessed immediately after islet isolation by fluorescent microscopy analysis. One thousand FDA-PI stained dispersed cells were counted per isolation. Control represents sham-operated animals where no direct brain damage was elicited; BD, brain death induced experimentally; and BD+Ex-4, brain death induced experimentally, followed by immediate administration of Exendin-4 (5μg/Kg of body weight). In *B*, percentages of viable cells out of total number of cells counted are shown. *C*, represents the percentages of dead cells in comparison with control animals. *D*, depicts representative fluorescence micrographs from the three experimental groups. Results are expressed as mean ± S.E.M (n = 3); **P*< 0.05.

Figure 2. Exendin-4 treatment promotes isolated islets function and ERK1/2 phosphorylation. Following isolation, islets rested for 3 hours before GSIS experiments were performed by static incubations as described in *Research Design and Methods*. For determination of ERK1/2 phosphorylation, whole-cell lysates from freshly isolated islets were immunoblotted for total and phospho ERK1/2. *A*, results from basal and high glucose incubations are represented in picograms of insulin per nanograms of DNA per hour. *B*, mean stimulation indexes are presented for all groups. *C*, Immunoblot analysis of total and phospho ERK1/2. *D-E*, relative quantification of ratios phospho ERK1 / total ERK1 and phospho ERK2 / total ERK2

respectively. All results are expressed as mean ± S.E.M (n = 3); #*P*< 0.1; **P*< 0.05; ***P*< 0.01.

Figure 3. Gene expression of pro-inflammatory cytokines in the pancreatic tissue. Relative mRNA expression of *IL-1β* (*A*) and *TNF-α* (*B*) were assessed in the pancreases of animals from all experimental groups by quantitative real time PCR. Results are expressed as mean fold change in comparison with control animals using *Actb* as endogenous control. Bars represent mean ± S.E.M (n = 5); *P < 0.05.

Figure 4. Gene expression of oxidative stress related genes in the pancreatic tissue. Relative mRNA expression of *Sod2* (*A*) and *Ucp2* (*B*) were assessed in the pancreases of animals from all experimental groups by quantitative real time PCR. Results are expressed as mean fold change in comparison with control animals using *Actb* as endogenous control. Bars represent mean ± S.E.M (n = 5); *P < 0.05; **P< 0.01).

Figure 5. Gene expression of ER stress related genes on freshly isolated islets. Relative mRNA expression of *Chop* (*A*) and *Bi*p (*B*) were assessed on freshly isolated islets from all experimental groups by quantitative real time PCR. Results are expressed as mean fold change in comparison with control animals using *Actb* as endogenous control. Bars represent mean ± S.E.M (n = 5); **P< 0.01).

Figure 6. Assessment of Caspase-3 activation on freshly isolated islets suggests that BD does not induce apoptotic cell death.

Whole-cell lysates from freshly isolated islets were immunoblotted for active

Caspase-3 with α-Tubulin as a loading control. *A*, immunoblot of three islet preparations from each experimental group is presented. Caspase-3 activation was below detection limit in all preparations. *B*, islets were cultured in the presence or absence of a cytokine mix (50 U/mL IL-1β, 1000 U/mL TNF-α and 100 U/mL IFNg) for 48 hours to serve as positive control for Caspase-3 activation.

Figure 7. BD does not affect apoptotic rate before or after isolation. *A*, Apoptosis prior to islet isolation was assessed *in situ* by immunohistochemistry to active Caspase-3. Quantification of pixel intensity is expressed as percentage of control animals. Bars represent mean ± S.E.M (n = 6). *B*, depicts representative Caspase-3 immunostaining micrographs. *C*, 1000 dispersed islet cells were counted per isolation and the presence of condensed or fragmented nuclei was estimated. Results are expressed as percentage of control. Bars represent mean ± S.E.M (n = 3). *D*, depicts representative Hoechst 33342 staining micrographs. White arrows indicate condensed or fragmented nuclei. *E*, depicts representative TUNEL staining micrographs from the three experimental groups and a positive DNAse I treated control. White arrows indicate positive stained nuclei. *F*, depicts representative insulin immunofluorescence stainings. *G*, results from Caspase-Glo assay are expressed as percentage of control animals. Bars represent mean ± S.E.M (n = 3).

Table 1. Oligonucleotide primers used for quantitative RT-PCR analyses

Target	Forward and reverse sequences
IL-1β	5' CAGAACATAAGCCAACAAGTGGTATT 3'
	5' CACAGGGATTTTGTCGTTGCT 3'
TNF-α	5' TGATCGGTCCCAACAAGGA 3'
	5' TGGGCTACGGGCTTGTCA 3'
Ccl2	5' CTGTGCTGACCCCAATAAGGA 3'
	5' ACTTGGTTCTGGTCCAGTTTTCTAA 3'
Tf	5' TTCGCACAAGCCGTGATTT 3'
	5' GGTGATGCTTTCTGGGCTCTT 3'
Chop	5' CATCCCCAGGAAACGAAGAG 3'
	5' AGCTAGGGATGCAGGGTCAA 3'
Bip	5' TCAAGGTCTACGAAGGTGAACGA 3'
	5' GTCAGATCAAATGTACCCAGAAGGT 3'
Sod2	5' GCCAAGGGAGATGTTACAACTCA 3'
	5' CCCCGCCATTGAACTTCA 3'
Ucp2	5' TCAACTGTACTGAGCTGGTGACCTA 3'
	5' GGAGGTCGTCTGTCATGAGGTT 3'
Actb	5' GACAGGATGCAGAAGGAGATTACTG 3'
	5' CTCAGGAGGAGCAATGATCTTGAT 3'

IL-1β, Interleukin-1 beta; *TNF-α*, Tumor necrosis factor; *Ccl2,* Chemokine (C-C motif) *l*igand 2; *Tf*, Tissue factor; *Chop*, C/EBP homologous protein; *Bip*, Immunoglobulin heavy-chain binding protein*; Sod2,* Superoxide dismutase 2; *Ucp2,* Uncoupling protein 2; *Actb,* β-actin.

Figure 1.

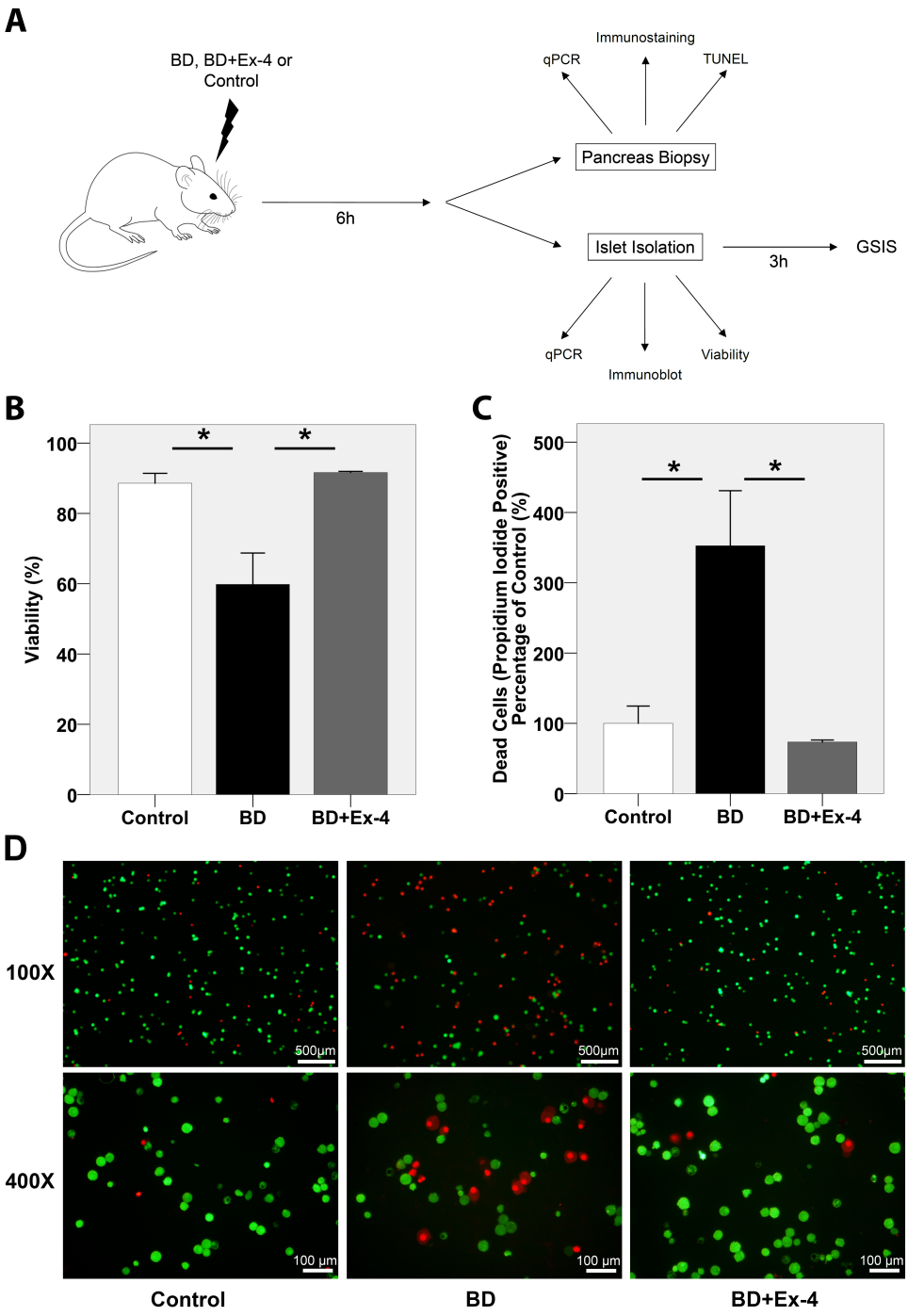

A
BD, BD+Ex-4 or Control
6h
Immunostaining
qPCR
TUNEL
Pancreas Biopsy
Islet Isolation
3h
GSIS
qPCR
Immunoblot
Viability
B
Viability (%)
100
80
60
40
20
0
*
*
Control
BD
BD+Ex-4
C
Dead Cells (Propidium Iodide Positive) Percentage of Control (%)
500
400
300
200
100
0
*
*
Control
BD
BD+Ex-4
D
100X
400X
500μm
100 μm
Control
BD
BD+Ex-4

Figure 2.

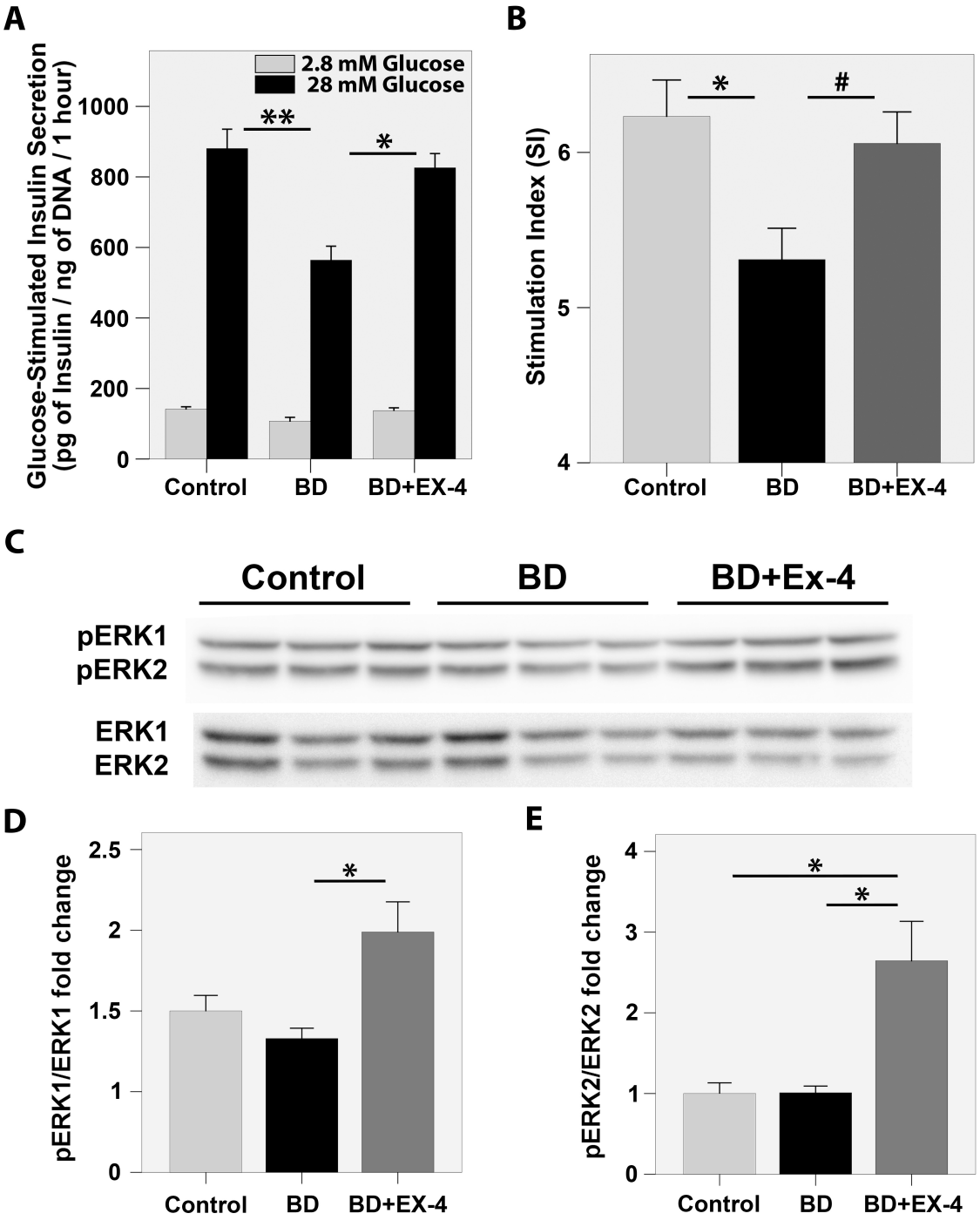

A
Glucose-Stimulated Insulin Secretion (pg of Insulin / ng of DNA / 1 hour)
2.8 mM Glucose
28 mM Glucose
1000
800
600
400
200
0
**
*
Control
BD
BD+EX-4
B
Stimulation Index (SI)
6
5
4
*
#
Control
BD
BD+EX-4
C
Control
BD
BD+Ex-4
pERK1
pERK2
ERK1
ERK2
D
pERK1/ERK1 fold change
2.5
2
1.5
1
0
*
Control
BD
BD+EX-4
E
pERK2/ERK2 fold change
4
3
2
1
0
*
*
Control
BD
BD+EX-4

Figure 3.

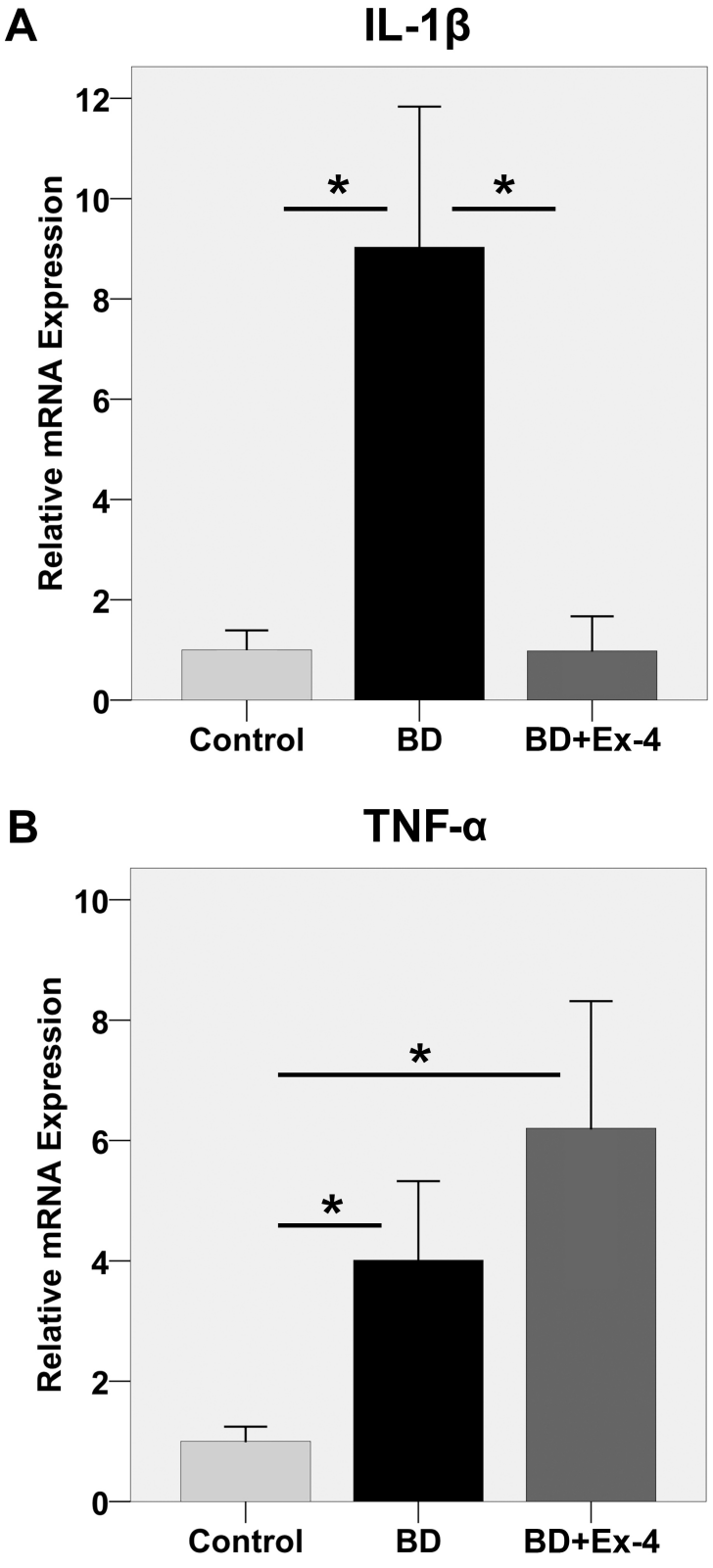

A
IL-1β
Relative mRNA Expression
12
10
8
6
4
2
0
*
*
Control
BD
BD+Ex-4
B
TNF-α
Relative mRNA Expression
10
8
6
4
2
0
*
*
Control
BD
BD+Ex-4

Figure 4.

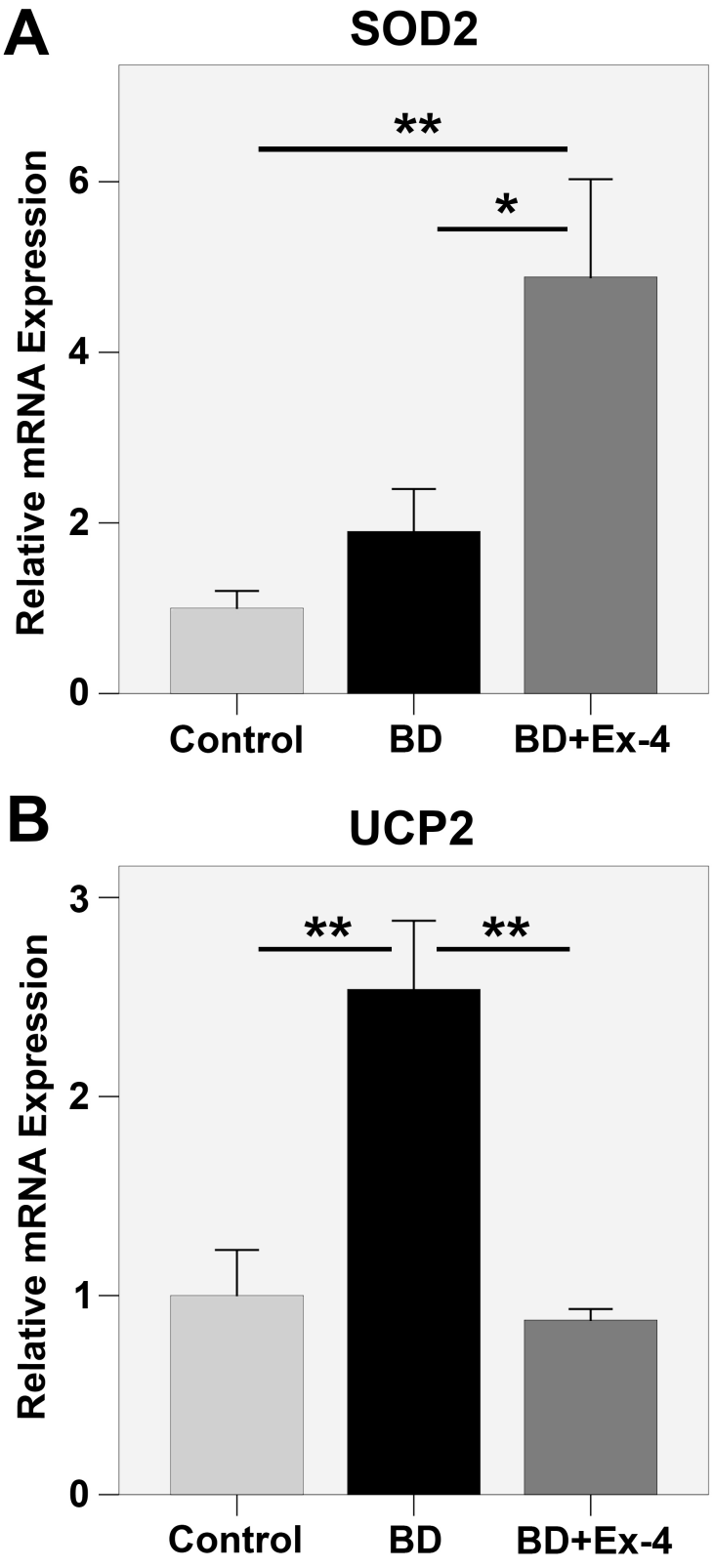

A
SOD2
Relative mRNA Expression
0
2
4
6
**
*
Control
BD
BD+Ex-4
B
UCP2
Relative mRNA Expression
0
1
2
3
**
**
Control
BD
BD+Ex-4

Figure 5.

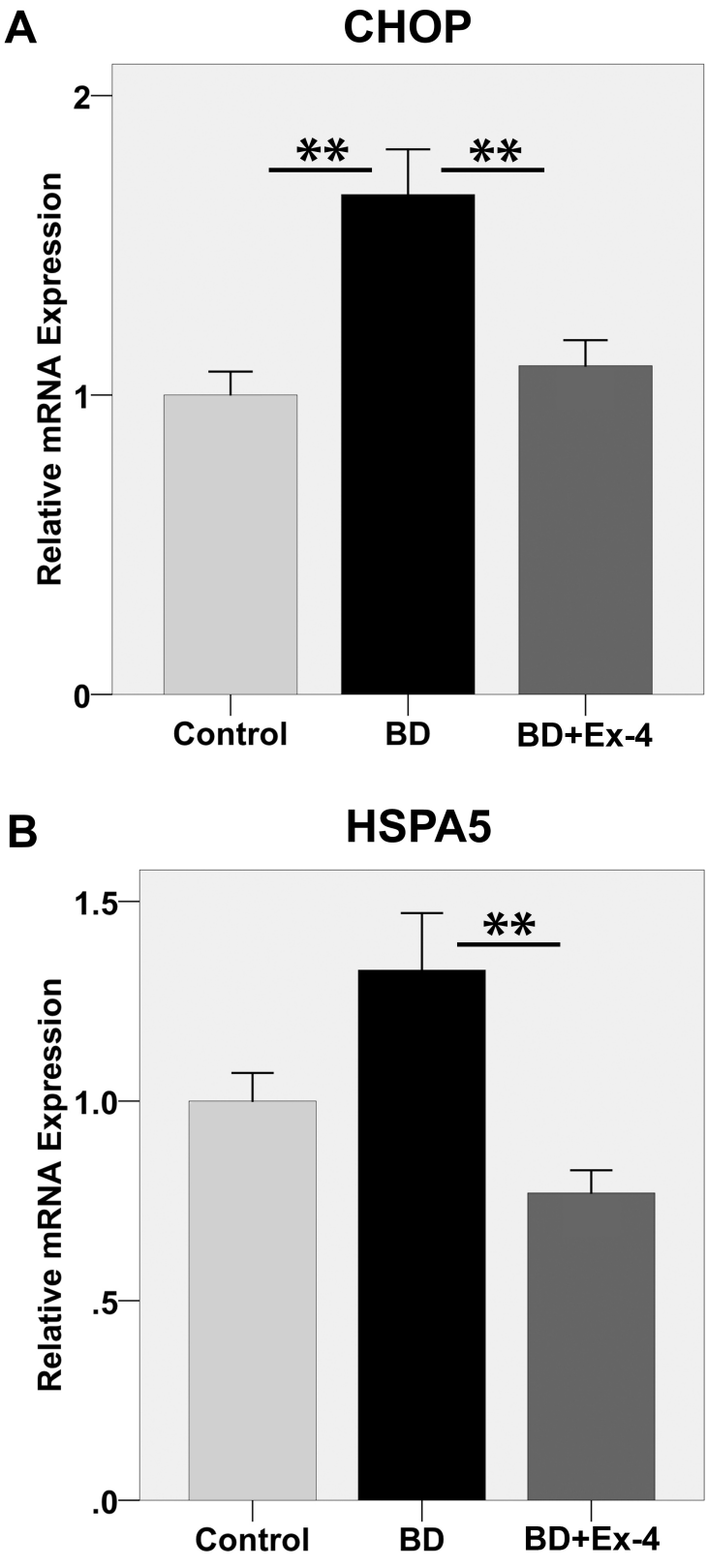

A
CHOP
Relative mRNA Expression
2
1
0
**
**
Control
BD
BD+Ex-4
B
HSPA5
Relative mRNA Expression
1.5
1.0
.5
.0
**
Control
BD
BD+Ex-4

Figure 6.

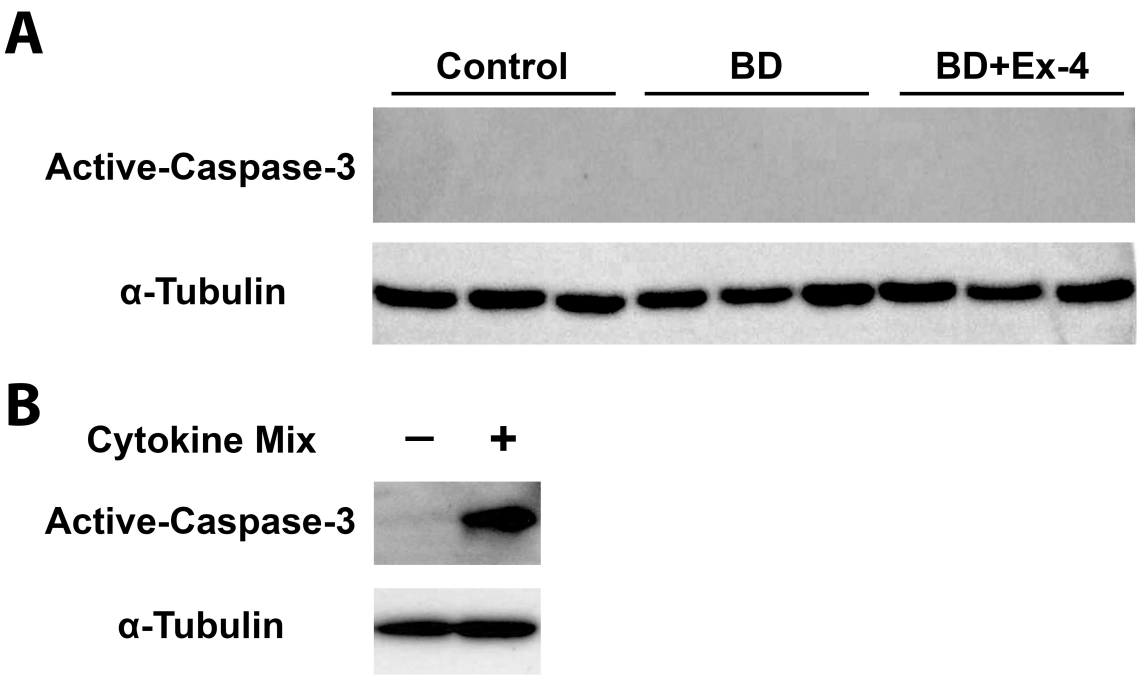
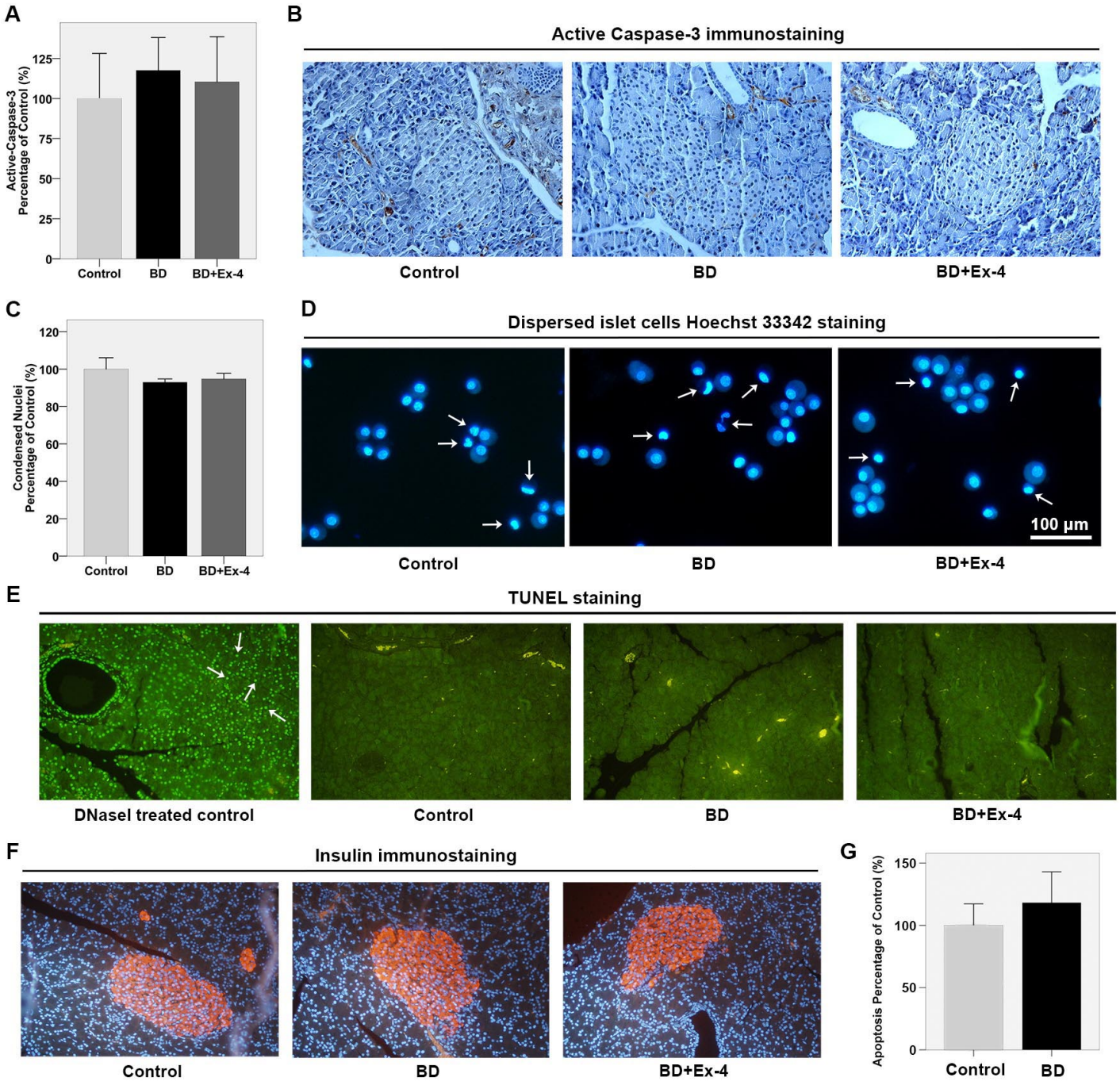

A
Control
BD
BD+Ex-4
Active-Caspase-3
α-Tubulin
B
Cytokine Mix
–
+
Active-Caspase-3
α-Tubulin

Figure 7.


A
Active-Caspase-3 Percentage of Control (%)
0
25
50
75
100
125
Control
BD
BD+Ex-4
B
Active Caspase-3 immunostaining
Control
BD
BD+Ex-4
C
Condensed Nuclei Percentage of Control (%)
0
20
40
60
80
100
120
Control
BD
BD+Ex-4
D
Dispersed islet cells Hoechst 33342 staining
100 µm
Control
BD
BD+Ex-4
E
TUNEL staining
DNaseI treated control
Control
BD
BD+Ex-4
F
Insulin immunostaining
Control
BD
BD+Ex-4
G
Apoptosis Percentage of Control (%)
0
50
100
150
Control
BD

PARTE 2

Exendin-4 attenuates brain death-induced liver damage in the rat

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Exendin-4 attenuates brain death-induced liver damage in the rat

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Key Words: brain death, liver transplantation, apoptosis, GLP-1 analogs

List of abbreviations

Actb, β-actin; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; Bcl-2, B-cell Lymphoma 2; BD, brain death; Ccl2, Chemokine (C-C motif) Ligand 2; DAB, diaminobenzidine tetrahydrochloride; ER, endoplasmic reticulum; Ex-4, Exendin-4; GLP1, Glucagon-Like Peptide-1; Hif1a, Hypoxia-Inducible Factor 1-alpha; HRP, horseradish peroxidase; Hspa5, Heat Shock 70 kDa Protein 5; Il1b; Interleukin-1 beta; Il6, Interleukin-6; LDH, lactate dehydrogenase; NAFLD, nonalcoholic fatty liver disease; NFκB, Nuclear Factor Kappa B; Sod2, Superoxide Dismutase 2; TBARS, thiobarbituric acid reactive substances; Tnf, Tumor Necrosis Factor; *TUNEL,* Terminal deoxynucleotidyl transferase dUTP nick end labeling; Ucp2, Uncoupling Protein 2.

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Conflicts of interests.

The authors declare no potential conflicts of interests relevant to this article.

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ABSTRACT

The majority of liver grafts destined to transplantation originate from brain-dead donors. However, significantly better post-transplantation outcomes are achieved when organs from living donors are used; suggesting that brain death (BD) causes irreversible damage to the liver tissue. Recently, Glucagon-like peptide-1 (GLP1) analogs were shown to possess interesting hepatic protection effects in different liver disease models. We hypothesized that donor treatment with the GLP1 analog Exendin-4 could alleviate BD-induced liver damage. A rat model of BD was employed in order to estimate BD-induced liver damage and Exendin-4's potential protective effects. Liver damage was assessed by biochemical determination of circulating hepatic markers. Apoptosis in the hepatic tissue was assessed by immunoblot and immunohistochemistry using an antibody that only recognizes the active form of Caspase-3. Gene expression changes in inflammation and stress response genes were monitored by quantitative Real Time PCR (qRT-PCR). Here, we show that Exendin-4 administration to the brain-dead liver donors significantly reduces levels of circulating aspartate aminotransferase (AST) and lactate dehydrogenase (LDH). This was accompanied by a remarkable reduction in hepatocytes apoptosis. In this model, BD caused up-regulation of Tumor Necrosis Factor (*Tnf*) and stress related genes, confirming previous findings in clinical and animal studies. **Conclusion –** Treatment of brain-dead rats with Exendin-4 reduced BD-induced liver damage. Further investigation is needed to determine the molecular basis of the observed liver protection. After testing in a randomized clinical trial, the inclusion of GLP1 analogs in the organ donor management might help to improve organ quality, maximize organ donation and possibly increase liver transplantation success rates.

INTRDUCTION

Organ transplantation is the best therapy option for patients with various end-stage organ diseases. Nevertheless, it is highly limited by organ unavailability. A promising alternative to overcome organ shortage is to maximize brain-dead organ donation (1). However, donor brain death (BD) is a catastrophic systemic event, which exerts a negative impact on donor organ quality. In the 1990s decade, kidney transplantation studies demonstrated that organs originated from living donors presented better survival rates than those derived from brain-dead donors (2, 3). Ever since, human and animal model research has evidenced BD-induced damage in various organs and tissues including kidneys, heart, lungs and pancreas (4-7). In the liver tissue, BD effects have been extensively investigated. Direct liver damage was demonstrated by increase of circulating hepatic markers following the experimental induction of BD in a rat model. This was accompanied by an increase in inflammatory parameters such as leukocyte infiltration and expression of adhesion molecules (8). Corroborating the view that BD causes direct liver damage, Van Der Hoeven et al. (9) showed that BD triggers apoptotic cell death of liver cells. After transplantation, livers from living donors show remarkably superior post-transplant survival when compared with livers from deceased donors (10, 11). Thus, amelioration of such deleterious effects caused by BD on the hepatic tissue has the potential to be translated into a new strategy to enlarge the utilization of marginal donor organs as well as improve hepatic transplantation outcomes.

Recently, anti-inflammatory and cytoprotective properties of Glucagon-like peptide-1 (GLP1) analogs, remarkably on pancreatic β-cells, but strikingly also in liver tissue, have been demonstrated (12-15). Therefore, we hypothesized that administration of the GLP1 analog Exendin-4 to the brain-dead donor, due to its

cytoprotective properties, might prevent liver tissue damage induced by BD. Here, we show for the first time that Exendin-4 administration following the establishment of BD in a rat model alleviates hepatocytes apoptosis and liver tissue damage.

MATERIALS AND METHODS

Animals

Male Wistar rats, fed standard laboratory diet ad libitum, weighting 300-350 g, were used throughout the study. All animals were kept in the animal facility of Hospital de Clínicas de Porto Alegre (Porto Alegre, Brazil) and were cared according to the guidelines for use and care of laboratory animals (16). The study was approved by the local ethics committee for the use of animals in research (project number 11-0623).

Experimental design

Animals were randomized in three experimental groups: Control, no central nervous system injury; BD, brain death induced experimentally; and BD+Ex-4, brain death induced experimentally, followed by immediate intraperitoneal administration of Exendin-4 (Exenatide; Eli Lilly, Indianapolis, IN, USA) 5μg/Kg of body weight. First, animals were anesthetized by a mixture of 2% isoflurane (Biochimico, Itatiaia, Brazil) in oxygen using a precision vaporizer (Takaoka, model 1415, Sao Paulo, Brazil). Then, they were intubated using a plastic cannula from a 16-gauge catheter (Abbocath®; Abbott Laboratories, Abbott Park, IL, USA) and mechanic ventilated using a volumetric ventilator (Harvard Rodent Ventilator, model 683; Harvard Apparatus Co., Millis, MA, USA) with a tidal volume of 2.5 mL and frequency of 85 strokes per minute. Arterial blood pressure was monitored continuously through cannulation of the left femoral artery, attached to a Biopac MP100 data acquisition

system (Biopac Systems Inc. Goleta, CA, USA). Brain death was induced using a modified version of the method previously published by Takada et al. (17). Briefly, a hole was drilled at the left front lateral region of the animal's skull to permit the passage of a No. 4 Fogarty catheter (Fogarty Arterial Embolectomy Catheter, 4F; Edwards Lifesciences, Irvine, CA, USA) without direct cerebral damage. The balloon was then rapidly inflated with 0.5 mL of saline for one minute. Animals in the control (sham-operated) group had the catheter inserted but not inflated. Maximally dilated pupils, apnea and absence of palpebral reflexes confirmed the BD condition. Body temperature was preserved at 37^0C by means of a heated dorsal table. Animals were maintained for six additional hours prior to cardiac puncture for total blood collection and liver biopsy.

Quantitative RT-PCR

RNA was extracted from liver tissue using RNeasy Mini Kit (Qiagen, Chatsworth, CA) and was reverse-transcribed using SuperScript® *VILO*™ cDNA Synthesis Kit (Invitrogen Life Technologies, Gaithersburg, MD, USA) according to manufacturer's protocols. Quantitative PCR was performed by monitoring the increase in fluorescence of Fast SYBR® Green Master Mix (Invitrogen Life Technologies, Gaithersburg, MD, USA) (18). Rat specific primer sequences for the genes Interleukin-1 beta (*Il1b*), Tumor Necrosis Factor (*Tnf*), Chemokine (C-C motif) Ligand 2 (*Ccl2*), Interleukin-6 *(Il6)*, Superoxide Dismutase 2 (*Sod2*), Uncoupling Protein 2 (*Ucp2*), Hypoxia-inducible factor 1-alpha (*Hif1a*), Heat Shock 70kDa Protein 5 (*Hspa5*), B-cell lymphoma 2 (*Bcl-2*) and β-actin (*Actb*) were designed using Primer Express 3.0 software (Life Technologies, Gaithersburg, MD, USA). Table 1 contains sequence information for all primers used. All the reactions were performed in a Viia™ 7 real time PCR system (Life Technologies, Gaithersburg, MD, USA).

Relative mRNA expression was calculated through the standard curve method (19), using β-actin as the reference gene. Quantitative RT-PCR specificity was determined using melting curve analyses and all primers generated amplicons that produced a single sharp peak during the analyses.

Immunoblot

Total protein extracts from frozen liver biopsies were resolved on 12% polyacrylamide gels, transferred to Immobilon®-PSQ membranes (Millipore, Billerica, MA, USA), and incubated with monoclonal antibody to Active Caspase-3 (Cell Signaling Technology, Beverly, MA, USA). Secondary antibody consisted of horseradish peroxidase conjugated goat anti-rabbit (Millipore, Billerica, MA, USA). Detection was performed using Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA, USA). For loading control, the membranes were stained with 0.1% Coomassie R-350 (GE Healthcare, Piscataway, NJ, USA), as recommended by Welinder et al. (20). Images were acquired in an ImageQuant LAS 500 (GE Healthcare, Piscataway, NJ, USA) digital imaging system and band densitometry analysis was performed using ImageJ 1.47v (National Institute of Health, USA).

Immunohistochemistry

Livers were removed, fixed in 10% phosphate buffered formalin, embedded in paraffin, sectioned (4 μm thick), and immunostained after deparaffinization and rehydration. Slides were incubated with Active Caspase-3 primary antibody (Cell Signaling Technology, Beverly, MA, USA) and then with a biotinylated secondary antibody, streptavidin-horseradish peroxidase conjugate, and diaminobenzidine tetrahydrochloride (DAB) (Dako Cytomation, Inc., Carpinteria, CA, USA). Slides were then counterstained with haematoxylin and images were

acquired using a Cool Snap-Pro CS (Media Cybernetics, Bethesda, MD, USA) camera attached to a Zeiss microscope (model AXIOSKOP-40; Carl Zeiss Microscopy GmbH, Jena, Germany).

Plasmatic measurements of hepatic damage markers

Approximately 4 mL of blood from each animal were collected into EDTA coated tubes (BD Vacutainer, Franklin Lakes, NJ, USA), through direct cardiac puncture. Plasma was recovered by centrifugation at 2000 RCF for 15 minutes at 4°C. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined by the kinetic ultraviolet method, lactate dehydrogenase (LDH) was measured by the enzymatic colorimetric method and alkaline phosphatase (ALP) by the kinetic colorimetric method. All measurements were performed in the ADVIA® 1800 Clinical Chemistry System (Siemens Healthcare Diagnostics Inc., Deerfield, Illinois, USA) according to International Federation of Clinical Chemistry. Results were expressed as IU/L.

Catalase and TBARS

For thiobarbituric acid reactive substances (TBARS) measurements, liver lysates were heated with thiobarbitutric acid (0.67%) and trichloroacetic acid (10%) for 10 minutes, as previously described by Draper and Hadley (21). After refrigeration and centrifugation, the ruddy product was quantified using spectrophotometry at 535 nm. The results were expressed as nmoles/mg of total proteins.

Catalase activity was assayed according to the method of Aebi (22), by measuring the absorbance decrease at 240 nm of 10 µl of liver lysates supernatants added to a reaction mixture (1 mL) of 20 mM H_2O_2, 0.1% Triton X-100, and 10 mM potassium phosphate buffer (pH 7.0). The results were expressed as IU/mg of total

proteins.

Statistical analysis

Variables are described as mean ± S.E.M. Variables with non-normal distribution were log-transformed before analyses. Differences across groups were tested using ANOVA with Tukey post hoc tests. Statistical significance was set at $P < 0.05$. All analyses were performed using SPSS 18 (IBM, Armonk, NY, USA).

RESULTS

Exendin-4 prevents BD-induced liver damage

Liver damage was assessed by measurement of hepatic markers (AST, ALT, LDH and ALP) in the plasma. Levels of AST and LDH were significantly higher in animals that underwent BD in comparison with control sham-operated rats. The treatment with Exendin-4 alleviated the impact of the BD on these two liver damage markers (AST - control 121 ± 19; BD 318 ± 26; BD+Ex-4 186 ± 31, IU/L, P <0.001; LDH - control 649 ± 171; BD 1463 ± 152; BD+Ex-4 872 ± 185, IU/L, P =0.007; Fig. 1*A* and 1*B*). ALT and ALP levels did not differ significantly between experimental groups (ALT - control 52 ± 4; BD 52 ± 6; BD+Ex-4 42 ± 7, IU/L, P =0.41; ALP - control 21 ± 4; BD 20 ± 5; BD+Ex-4 17 ± 5, IU/L, P =0.85; Fig. 1*C* and 1*D*).

In order to assess the apoptotic rate in the hepatic tissue, the activation status of Caspase 3 was determined both by immunoblot and immunohistochemistry. Total liver protein extracts from animals of each experimental group were subjected to immunoblot analysis using an antibody that only binds to the active form of Caspase-3. A significant increase in the levels of active Caspase-3 was observed in the livers of BD animals as compared to control animals and, interestingly, Exedin-4 administration prevented against such apoptosis induction (Fig. 2*A* and Fig. 2*B*). *In*

situ Caspase-3 activity was assessed by immunohistochemical staining of the active form of Caspase-3 in liver sections. Tissue derived from BD animals clearly presented a greater number of active-Caspase-3 stained cells. Again, administration of Exendin-4 was able to protect liver tissue against BD-induced apoptosis (Fig. 2*C*).

In order to assess if BD-induced liver apoptosis occurs because of a reduction in the anti-apoptotic gene *Bcl-2,* we measured gene expression quantities of this gene in liver samples. *Bcl-2*, however, did not differ between experimental groups, suggesting that BD-induced apoptosis does not involve modulation of this specific anti-apoptotic gene (Fig. 2*D*).

BD induces gene expression of inflammation and stress related genes

Many studies showed an increase of hepatic inflammation following BD (8, 23, 24). Aiming at evaluating a possible effect of Exendin-4 in the modulation of BD-induced liver inflammation, we assessed gene expressions of four important pro-inflammatory cytokines, *Tnf*, *Il1b*, *Il6* and *Ccl2* in the hepatic tissue. Inflammation in response to BD was evidenced by a significant increase in the hepatic expression of *Tnf*. Treatment with Exendin-4 did not change *Tnf* expression in comparison with non-treated BD animals (Fig. 3*A*). Expression levels of *Il1b*, *Il6* and *Ccl2* were not affected by BD and did not change significantly between experimental groups (Fig. 3*B-D*).

Next, we evaluated hepatic gene expression of stress related genes. We selected *Sod2* and *Ucp2*, which are known to be upregulated in situations of increased oxidative stress. In addition, the transcription factor *Hif1a* was also analyzed because of its responsiveness to hypoxia, which possibly occurs in the hepatic tissue after BD due to sub-optimal blood perfusion. Lastly, gene expression of *Hspa5,* an

endoplasmic reticulum (ER) resident molecular chaperone that mediates protein folding and it is induced when unfolded proteins accumulate in the ER lumen during ER stress conditions, was also assessed. Quantitative RT-PCR analyses evidenced a significant induction in the gene expression of these four genes in the liver tissue of rats that underwent BD. No significant differences were found between those animals and their Exendin-4 treated counterparts (Fig. 4*A-D*). In addition, liver extracts were tested for Catalase activity and TBARS production, and results are presented in Fig. 5*A* and Fig. 5*B*, respectively. No significant differences in these oxidative stress markers in response to BD were evident.

DISCUSSION

In our experimental model, BD caused an increase of liver damage markers in the circulation. Furthermore, liver tissue apoptosis was also induced in the same experimental conditions. These outcomes were accompanied by an increase in hepatic expression of the pro-inflammatory cytokine *Tnf* and stress related genes. Interestingly, Exedin-4 treatment after the induction of BD significantly decreased liver tissue damage and apoptosis.

Liver tissue damage and apoptosis after BD has previously been demonstrated in different animal models, including rodents and pigs (8, 25). In order to confirm those findings and test the effects of Exendin-4 administration in our experimental system, both direct and indirect evidence of hepatic impairment were investigated. Plasma levels of AST, ALT and LDH are largely used as indicators of general liver cell dysfunction. AST and LDH were increased in our BD model, whereas ALT was unaltered. This is in agreement with the study of Van der Hoeven et al. (8), in which AST and LDH, but not ALT, were elevated following the induction of experimental

BD. In our model, both AST and LDH levels were significantly reduced upon treatment with one single dose of Exendin-4 following BD establishment, indicating the protective potential of the drug against BD-induced liver dysfunction. We also assessed the levels of ALP, which is a general marker of damage to biliary duct cells; however, its levels were unaltered across the experimental groups, suggesting that BD probably does not cause direct damage to these particular hepatic structures. Altogether, data presented in this study support the idea that BD can stimulate apoptotic cell death of the liver tissue, and most important, that treatment with Exendin-4 notably protects liver cells against such BD-induced damage in rats (Figs. 1 and 2).

Several studies have previously demonstrated the protective potential of GLP-1 analogs for different liver diseases and insults. For instance, Exendin-4 protected liver cells against apoptosis induction in response to ischemic injury (26). On this particular study, Exendin-4 administration in the perioperative course acutely prevented liver damage, highlighting the potential of this drug to protect the liver tissue in a clinically relevant setting. Interestingly, Exendin-4 could also reverse hepatic steatosis in *ob*/*ob* mice (27). In addition, GLP-1 receptor stimulation was able to promote hepatic lipid oxidation in rat hepatocytes originated from high fat diet fed animals, suggesting a possible use for GLP-1 analogs in the improvement of hepatic insulin resistance in patients with nonalcoholic fatty liver disease (NAFLD) (15), reviewed in (28). Hepatic oxidative stress and inflammation have both been shown to be attenuated by GLP-1 analogs in different settings. Exendin-4 reduced TBARS production in the liver of *ob*/*ob* mice (27), and Liraglutide suppressed the hepatic expression of Tnf and Nuclear Factor Kappa B (NFκB) in two different mice models of NAFLD (29).

This prompted us to investigate if Exendin-4 treatment could modulate oxidative stress and inflammation markers associated with BD in our model. Our gene expression analysis supports previous findings that inflammation and oxidative stress take place in the hepatic tissue following the establishment of BD (30, 31). This can be concluded from our quantitative RT-PCR data, which demonstrate a significant increase of *Tnf* expression in BD-animals in comparison with sham-operated control counterparts. Furthermore, genes responsive to oxidative stress, hypoxia and ER stress are also up-regulated in our BD model, corroborating previous findings and adding new evidence for BD-induced hepatic dysfunction. The results of this study, however, cannot clarify the specific mechanisms by which Exendin-4 promote hepatic protection against BD-induced damage, since inflammation and stress related genes did not change significantly following BD-donor treatment with Exendin-4. Future studies are needed in order to unveil the molecular mechanisms involved in this process.

In this study, we provide evidence that Exendin-4 administration to brain dead rats reduces BD-induced liver damage. Our findings suggest a potential usage for Exendin-4 as a protective agent for the liver when administered to the deceased organ donor prior to organ procurement. Noteworthy, using the same BD model presented here, we have recently shown that donor treatment with Exendin-4 increases viability and function of pancreatic islets after isolation (32). Thus, introduction of this GLP-1 analog in the clinical practice might promote not only liver, but also pancreatic islet transplantation outcomes. Effects of Exendin-4 on other organs remain to be evaluated. After thorough evaluation in a randomized clinical trial, incorporation of this strategy in the clinical setting might add to the ongoing effort to maximize brain-dead liver donation and possibly improve current graft survival rates.

Authors' Contributions.

R.C. designed the study, researched data, and wrote the manuscript. N.E.L., A.L.D., L.A.B. and J.R.O researched data. A.C.B. and C.B.L. designed the study, contributed to discussion, and reviewed the manuscript. D.C. designed the study and reviewed/edited the manuscript. R.C. and D.C. are joint guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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Figure Legends

Figure 1. Biochemical measurements of circulating liver damage markers.

Levels of AST (*A*), LDH (*B*), ALT (*C*) and ALP (*D*) were measured in plasma samples of animals from all experimental groups. Bars represent mean IU/L ± S.E.M (n = 6); **P=0.007, ***P<0.001. AST, aspartate aminotransferase; LDH, lactate dehydrogenase; ALT, alanine aminotransferase; ALP, alkaline phosphatase.

Figure 2. Ex-4 protects against BD-induced liver tissue apoptosis.

A, Protein extracts from frozen liver biopsies originated from 3 animals of each experimental group were immunoblotted for active Caspase-3 and 0.1% Coomassie R-350 staining was used as loading control. *B*, Band densitometry analysis was performed using ImageJ 1.47v. Results are expressed in arbitrary units in relation to control. Bars represent mean ± S.E.M (n = 3); **P=0.005. Liver sections were subjected to immunohistochemical analysis to detect active Caspase-3. *C*, depicts representative micrographs from all the experimental groups. Arrows indicate active Caspase-3 positive cells. Bar represents 200 μm. *D,* Relative mRNA expression of *Bcl2* was assessed in the liver tissue of animals from all experimental groups by quantitative real time PCR. Results are expressed as mean fold change in comparison with control animals using *Actb* as endogenous control. Bars represent mean ± S.E.M (n = 6); P=0.98.

Figure 3. Gene expression of pro-inflammatory cytokines in the liver tissue. Relative mRNA expression of *Tnf* (*A*), *Il1b* (*B*), *Il6* (*C*) and *Ccl2* (*D*) were assessed in the liver

tissue of animals from all experimental groups by quantitative real time PCR. Results are expressed as mean fold change in comparison with control animals using *Actb* as endogenous control. Bars represent mean ± S.E.M (n = 6); *P=0.03.

Figure 4. Gene expression of stress related genes in the liver tissue.

Relative mRNA expression of *Sod2* (*A*), *Ucp2* (*B*) and *Hspa5* (*C*) were assessed in the liver tissue of animals from all experimental groups by quantitative real time PCR. Results are expressed as mean fold change in comparison with control animals using *Actb* as endogenous control. Bars represent mean ± S.E.M (n = 6); *P=0.01, **P=0.003, ***P<0.001.

Figure 5. Biochemical measurement of oxidative stress markers in the liver tissue

The production of thiobarbituric acid reactive substances (TBARS) (*A*) and the activity of Catalase (*B*) were measured in liver tissue lysates of animals from all the experimental groups as described in Materials and Methods. Results are expressed in the indicated units. Bars represent mean ± S.E.M (n = 6).

Table 1. Oligonucleotide primers used for quantitative RT-PCR analyses

Target	Forward and Reverse sequences
Il1b	5' CAGAACATAAGCCAACAAGTGGTATT 3'
	5' CACAGGGATTTTGTCGTTGCT 3'
Tnf	5' TGATCGGTCCCAACAAGGA 3'
	5' TGGGCTACGGGCTTGTCA 3'
Ccl2	5' CTGTGCTGACCCCAATAAGGA 3'
	5' ACTTGGTTCTGGTCCAGTTTTCTAA 3'
Il6	5' AAGTCGGAGGCTTAATTACATATGTTC 3'
	5' TCATCGCTGTTCATACAATCAGAA 3'
Sod2	5' GCCAAGGGAGATGTTACAACTCA 3'
	5' CCCCGCCATTGAACTTCA 3'
Ucp2	5' TCAACTGTACTGAGCTGGTGACCTA 3'
	5' GGAGGTCGTCTGTCATGAGGTT 3'
Hif1a	5' GAAGCGAAAAATGGAACATGATG 3'
	5' CCTGGTTGCTGCAGTAACGTT 3'
Hspa5	5' TCAAGGTCTACGAAGGTGAACGA 3'
	5' GTCAGATCAAATGTACCCAGAAGGT 3'
Bcl2	5' GGCATCTGCACACCTGGAT 3'
	5' GGGCCATATAGTTCCACAAAGG 3'
Actb	5' GACAGGATGCAGAAGGAGATTACTG 3'
	5' CTCAGGAGGAGCAATGATCTTGAT 3'

Il1b, Interleukin-1 beta; *Tnf*, Tumor Necrosis Factor; *Ccl2,* Chemokine (C-C motif) Ligand 2; *Il6,* Interleukin-6*; Sod2,* Superoxide Dismutase 2; *Ucp2,* Uncoupling Protein 2; *Hif1a,* Hypoxia-Inducible Factor 1-alpha*; Hspa5,* Heat Shock 70kDa Protein 5; *Bcl-2,* B-cell lymphoma 2; *Actb,* β-actin.

Figure 1.

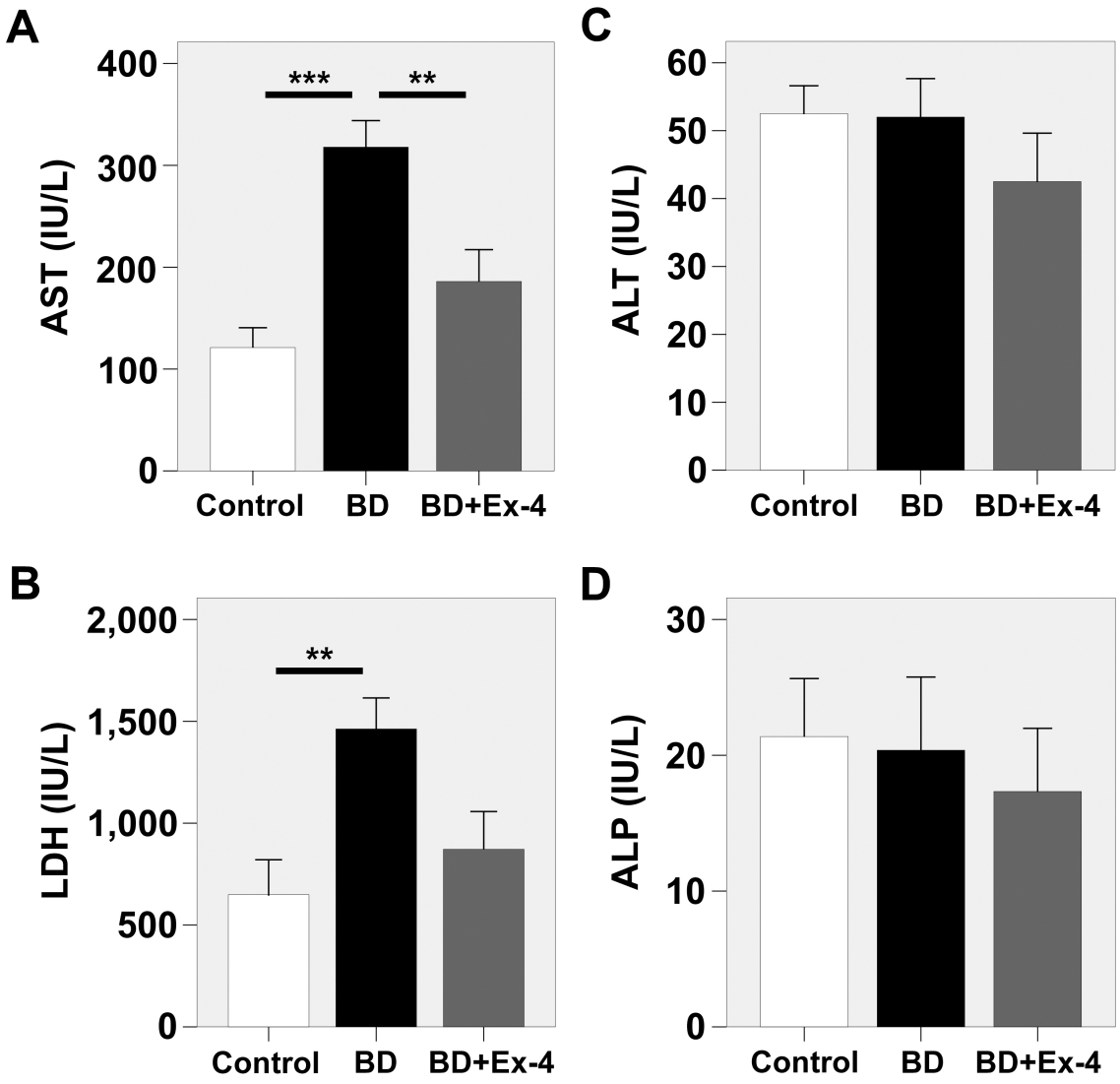

A
AST (IU/L)
400
300
200
100
0
***
**
Control
BD
BD+Ex-4
C
ALT (IU/L)
60
50
40
30
20
10
0
Control
BD
BD+Ex-4
B
LDH (IU/L)
2,000
1,500
1,000
500
0
**
Control
BD
BD+Ex-4
D
ALP (IU/L)
30
20
10
0
Control
BD
BD+Ex-4

Figure 2.

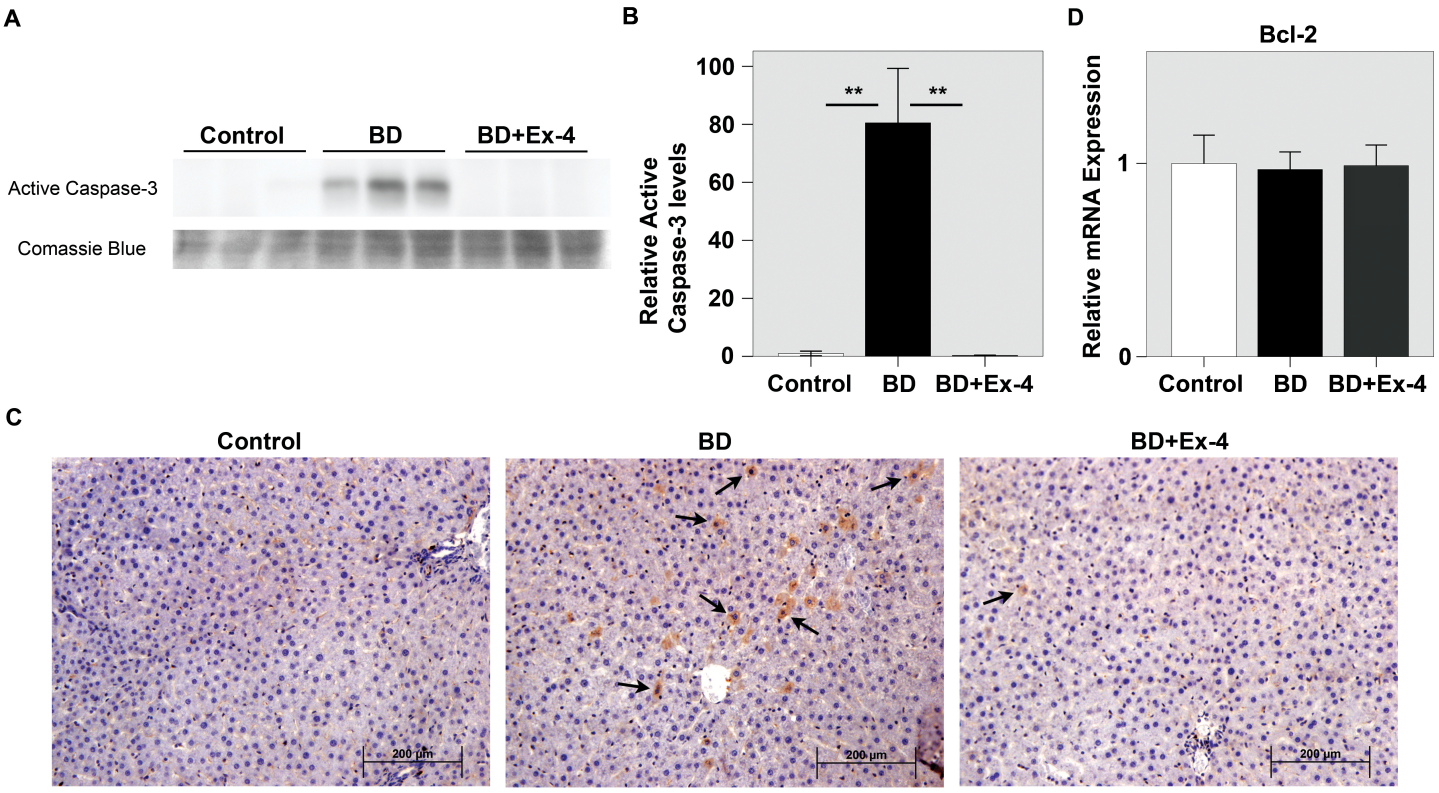

A
Control
BD
BD+Ex-4
Active Caspase-3
Comassie Blue
B
Relative Active Caspase-3 levels
100
80
60
40
20
0
**
**
Control
BD
BD+Ex-4
D
Bcl-2
Relative mRNA Expression
1
0
Control
BD
BD+Ex-4
C
Control
BD
BD+Ex-4
200 μm
200 μm
200 μm

Figure 3.

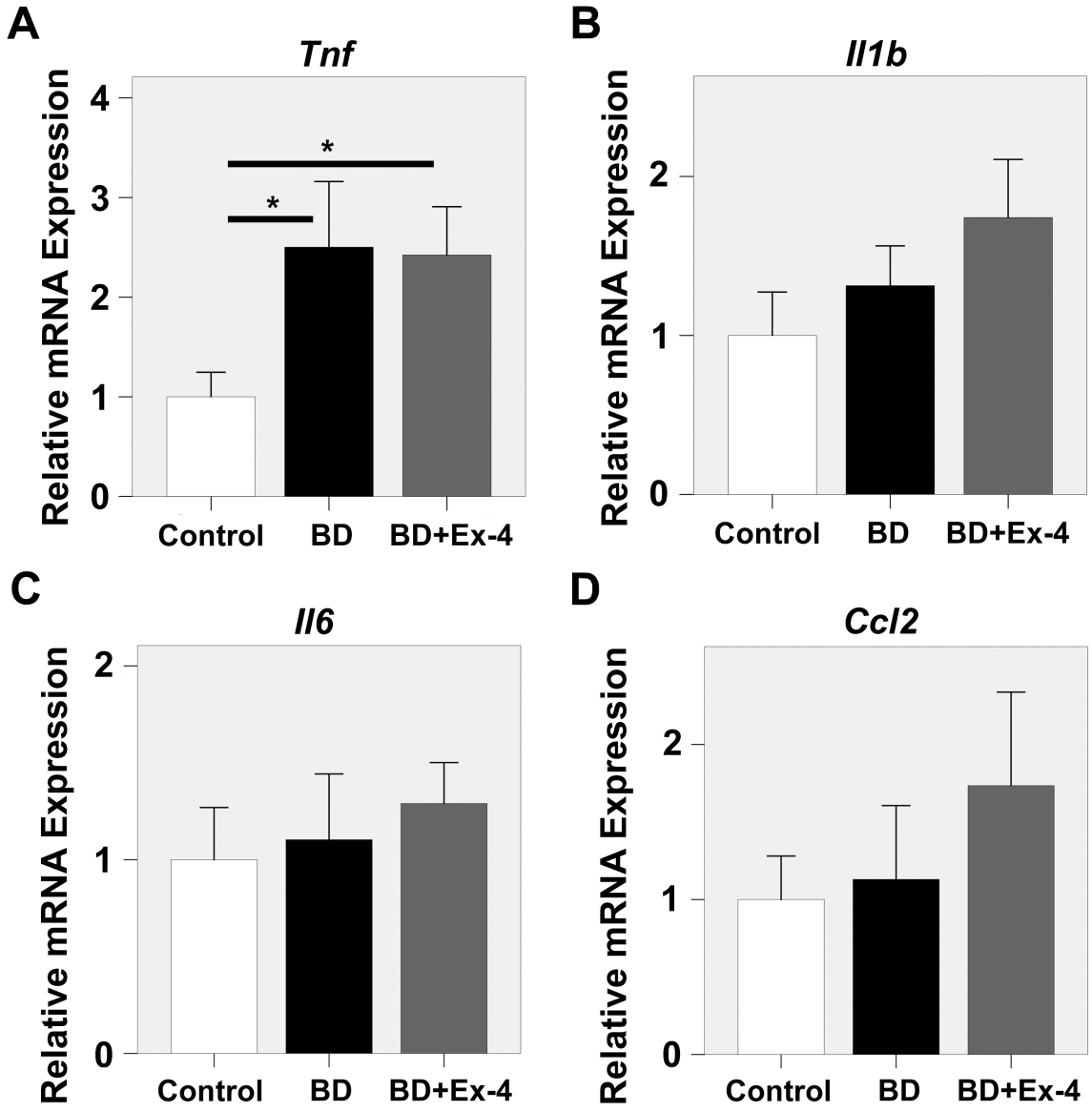

A
Tnf
Relative mRNA Expression
0
1
2
3
4
*
*
Control
BD
BD+Ex-4
B
Il1b
Relative mRNA Expression
0
1
2
Control
BD
BD+Ex-4
C
Il6
Relative mRNA Expression
0
1
2
Control
BD
BD+Ex-4
D
Ccl2
Relative mRNA Expression
0
1
2
Control
BD
BD+Ex-4

Figure 4.

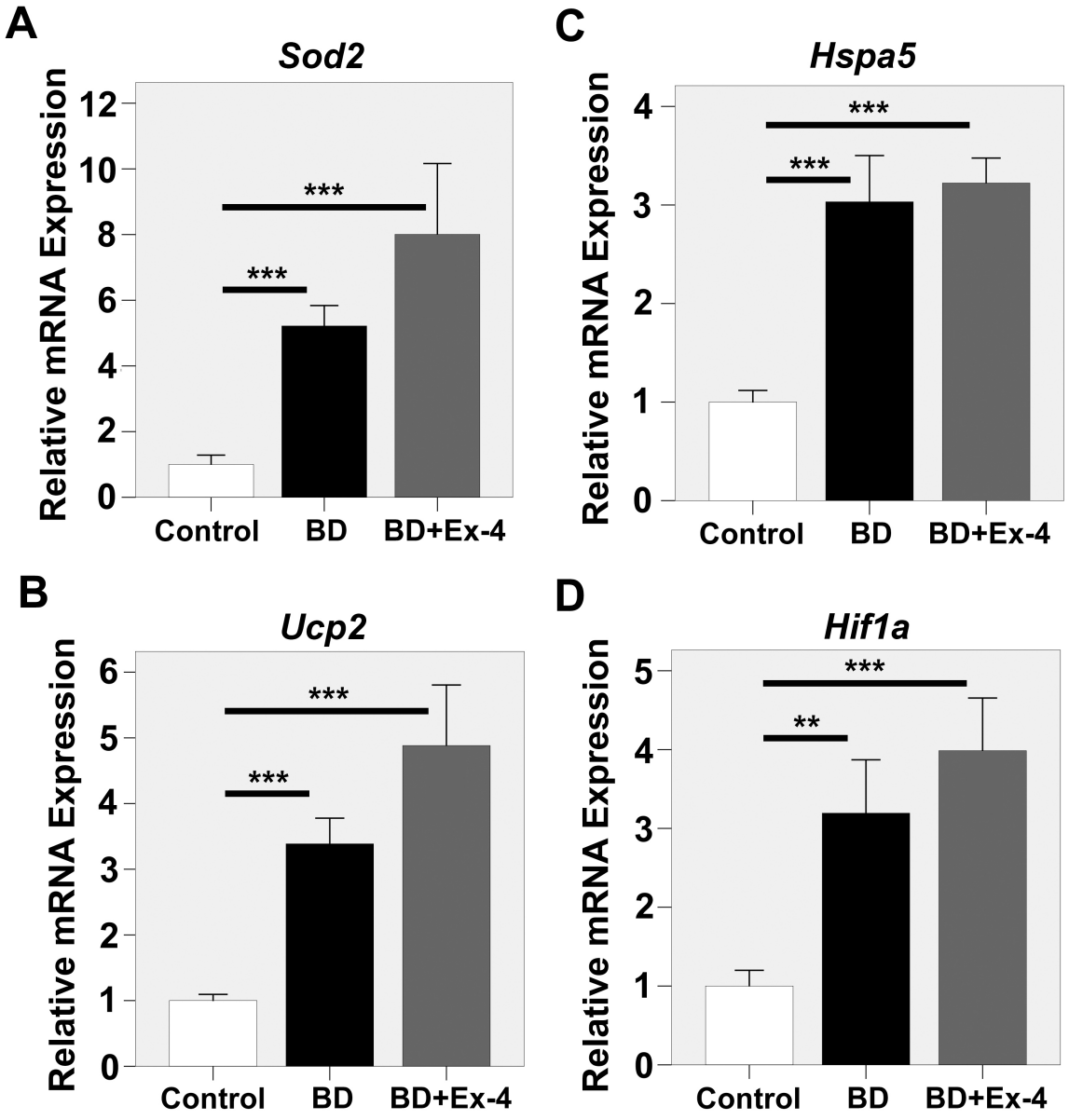
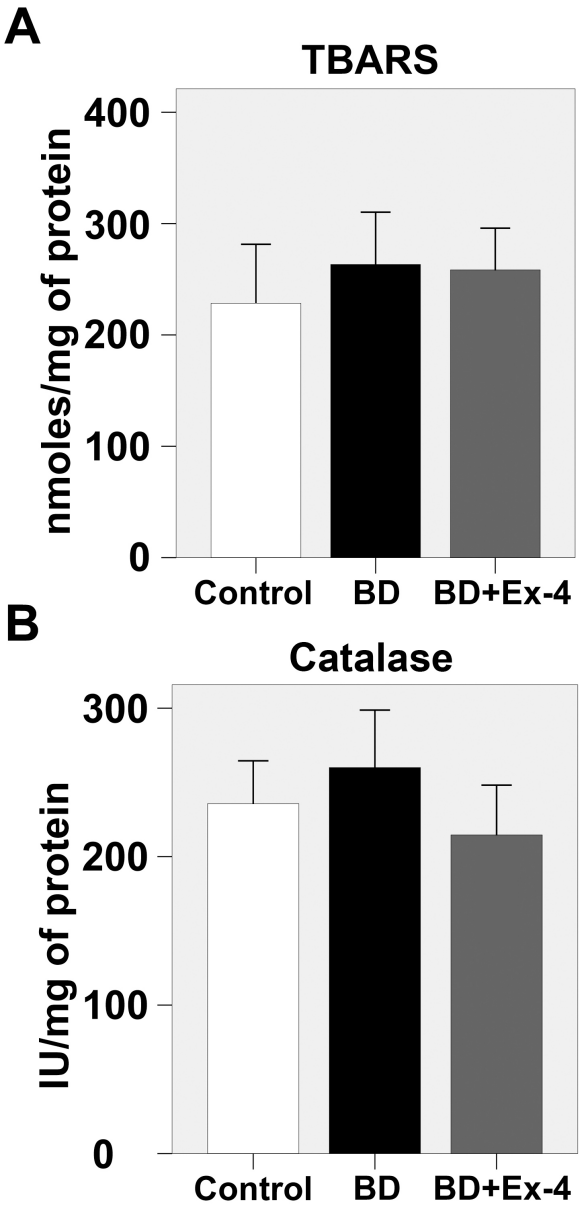

A
Sod2
Relative mRNA Expression
12
10
8
6
4
2
0
***
***
Control
BD
BD+Ex-4
C
Hspa5
Relative mRNA Expression
4
3
2
1
0
***
***
Control
BD
BD+Ex-4
B
Ucp2
Relative mRNA Expression
6
5
4
3
2
1
0
***
***
Control
BD
BD+Ex-4
D
Hif1a
Relative mRNA Expression
5
4
3
2
1
0
***
**
Control
BD
BD+Ex-4

Figure 5.


A
TBARS
nmoles/mg of protein
400
300
200
100
0
Control
BD
BD+Ex-4
B
Catalase
IU/mg of protein
300
200
100
0
Control
BD
BD+Ex-4

PARTE 3

GLP-1 receptor signaling promotes β-cell glucose metabolism *via* mTOR-dependent HIF-1α activation

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GLP-1 receptor signaling promotes β-cell glucose metabolism *via* mTOR-dependent HIF-1α activation

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Word count: 3822

ABSTRACT

Glucagon-like-peptide-1 (GLP-1) effectively promotes insulin secretion from pancreatic β-cells in a glucose dependent manner. Several pathways have been demonstrated to mediate insulin secretion in response to GLP-1 receptor (GLP-1R) ligation by rapid, kinase phosphorylation-dependent, but gene expression-independent mechanisms. Here we report that stimulation with the GLP-1 analogue, Exendin-4, stimulates β-cell glucose metabolism through up-regulation of glycolytic gene expression. The subsequent increase in glycolysis permits higher rates of ATP generation, indispensable for stimulus-secretion coupling. Maximal mitochondrial respiration and capacity, two parameters that are rate limited by the glycolytic flux, are significantly elevated in response to Exendin-4 stimulation. GLP-1R signaling has previously been shown to induce translation of Hypoxia-Inducible Factor 1 alpha (HIF-1α) in β-cells by activation of the mammalian Target of Rapamycin (mTOR) pathway. In our model, depletion of HIF-1α impaired the effects of Exendin-4 on glucose metabolism, while pharmacological inhibition of Phosphoinositide 3-kinase (PI3K) or mTOR completely abolished such effects. Considering the central role of glucose catabolism for insulin secretion, our findings suggest that GLP-1 actions on β-cells include increased glucose metabolism. Moreover, our data reveal novel aspects of GLP-1 stimulated insulin secretion involving *de novo* gene expression.

INTRODUCTION

Insulin release from pancreatic β-cells is a complex and highly controlled process (1). It is tightly regulated by stimulus-secretion coupling, whereby glucose catabolism within the β-cell is the primary signal for secretion. ATP generated through glycolysis and mitochondrial respiration brings about closure of ATP-sensitive K^+ channels, subsequently causing membrane depolarization, leading to activation of voltage-dependent Ca^{2+} channels (VDCCs), Ca^{2+} influx and exocytosis of insulin vesicles (2). Therefore, normal responses to carbohydrate containing meals or drinks involve increasing glucose metabolism in β-cells, which subsequently enhances insulin secretion. However the latter responses are impaired in type 2 diabetes (T2D), where β-cell dysfunction plays a major role (3). Thus, any treatment capable of promoting glucose metabolism and insulin secretion from β-cells may result in clinical benefit from improvement in body glucose homeostasis.

The incretin hormone Glucagon-like peptide-1 (GLP-1) physiologically enhances glucose-dependent insulin secretion from pancreatic β-cells (4). Clinically, GLP-1 analogues improve glucose stimulated insulin secretion (GSIS) in T2D patients, thus ameliorating hyperglycemia (5-7). GLP-1 exerts its cellular actions by binding to a G-protein coupled receptor (GLP-1R), expressed on the surface of many cells including β-cells, which, upon stimulation, leads to activation of adenylyl cyclase thus increasing intracellular levels of cAMP (4). cAMP directly activates protein kinase A (PKA) and cAMP-regulated guanine nucleotide exchange factor 2 (Epac2), that act in concert to generate downstream signals that result in increased insulin secretion (8). Mechanisms

include promotion of closure of ATP sensitive K^+ channels, facilitating the opening of VDCCs, inhibiting membrane repolarization *via* Kv channels and facilitating Ca^{2+}-induced Ca^{2+} release (CICR) from cytoplasmic storage sites such as the endoplasmic reticulum (9-13), reviewed in (14).

All known mechanisms of GLP-1-induced insulin secretion depend on glucose metabolism. Thus, it is attractive to hypothesize that GLP-1 signaling could enhance flux and oscillation through the glycolytic pathway to generate metabolic stimulus-secretion factors. Supporting this hypothesis, GLP-1 was shown to stimulate Hypoxia-Inducible Factor 1 (HIF-1) activity *via* induction of the mammalian Target of Rapamycin (mTOR) in β-cells (15). HIF-1 is a heterodimeric transcriptional factor composed of two subunits, HIF-1α and HIF-1β [also known as Aryl hydrocarbon Receptor Nuclear Translocator (ARNT)] (16). It induces metabolic reprogramming in response to hypoxia and growth factor signaling (17; 18), partly by promoting transcriptional activation of glycolytic genes (19). HIF-1α and HIF-1β are crucial for cell survival during hypoxia (20), however, their importance for normal β-cell function in normoxic conditions has been reported (21; 22).

Here we show that, chronic stimulation of the GLP-1R increases glycolysis and ATP production in β-cells through transcriptional activation and expression of glycolytic genes. Pharmacological inhibition of the PI3K/mTOR pathway abolished such effects, suggesting that the metabolic actions of GLP-1 occur *via* mTOR activation. In addition, we observed that HIF-1α protein levels accumulate downstream of mTOR in response to GLP-1 signaling whereas we also demonstrated that depletion of HIF-1α impaired GLP-1 action on both glycolysis and transcriptional regulation of glycolytic genes. Based on

these findings, we propose that chronic exposure to GLP-1 signaling promotes metabolic reprograming of β-cells *via* activation of the HIF-1 transcriptional program

RESEARCH DESIGN AND METHODS

Animals. β-Hif1a-null mice were generated using the Cre-lox system, as described in (21). Mice, with floxed HIF-1α (38), were bred with mice expressing Cre-recombinase, under control of the rat insulin promoter. All animals were cared according to the guidelines for use and care of laboratory animals (39). The study was approved by the Western Sydney Local Health District Animal Ethics Committee (WSLHD AEC) for the use of animals in research (protocol 4224.06.14).

Reagents. All chemicals were purchased from Sigma-Aldrich, unless indicated otherwise. Rabbit polyclonal antibody against HIF-1α was obtained from (Novus Biologicals, Littleton, CO, USA, #NB100-449), while phosphorylated mTOR S2448 and β-actin were obtained from (Cell Signaling Technology, Beverly, MA, USA, #2971 and #4970 respectively). Secondary antibodies consisted of horseradish peroxidase conjugated goat anti-rabbit IgG (Agilent's Dako, Glostrup, Denmark). Torin 1, LY294002 and Forskolin were purchased from (ApexBio, Houston, TX, USA).

Cell culture. Clonal insulin-secreting BRIN-BD11 cells were maintained in RPMI 1640 medium containing 11.1 mM glucose, supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 0.1 mg/ml streptomycin, pH 7.4, as described previously (40). Cells were maintained in T75 sterile tissue culture flasks at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air.

Islet isolation. Islets were isolated from mice as previously described (22). After purification, islets were cultured overnight at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 in RPMI 1640 medium supplemented with 10% FBS, 100 units/ml penicillin, 0.1 mg/ml streptomycin and 25 mM HEPES. Islets were subsequently hand picked and placed into individual wells of a 96-well plate for glucose consumption and total ATP content experiments.

Glucose consumption, lactate production and total ATP content assays. After pre-conditioning in the presence or absence of 50 nM Exendin-4 for 18 hours, BRIN-BD11 cells or isolated mice islets were cultured in RPMI containing 20 mM of glucose in the absence of Exendin-4 for additional 24 hours. Tissue culture media supernatants from the last 24 hours of culture were collected and assayed by Amplex Red Glucose/Glucose Oxidase Assay Kit (Life Technologies, Gaithersburg, MD, USA) and Lactate Assay kit (Sigma-Aldrich) to measure total glucose and lactate respectively. Cells were lysed with RIPA buffer (Astral Scientific, Sydney, Australia) and total DNA quantified using Quanti-iT PicoGreen (Life Technologies, Gaithersburg, MD, USA). Results were reported as nmoles of glucose or lactate and normalized by total DNA or number of islets. For total ATP content measurements, BRIN-BD11 cells or islets were lysed with CellTiter-Glo® Reagent (Promega Corporation, Madison, WI, USA) and total ATP determined. All measurements were performed according to manufacturer's instructions.

Extracellular flux analysis. Bioenergetics was determined using the XF^e96 Extracellular Flux Analyzer (Seahorse Bioscience, North Billerica, MA). BRIN-BD11 cells were seeded into specialized 96-well plates at a density of 10^4 cells/well and allowed to adhere overnight. Cell density was previously optimized so that OCR and

ECAR measurements met the manufacturer's criteria. Then, cells were pre-conditioned in the presence or absence of 50 nM Exendin-4 for additional 18 hours. Culture medium was changed to serum-free Dulbecco's Modified Eagle's Medium, containing 1 mM sodium pyruvate, without glucose and sodium bicarbonate (Seahorse base media). Plates were then incubated for one hour at 37 °C in a CO_2-free atmosphere. Basal OCR (indicator of mitochondrial respiration) and ECAR (indicator of lactate production or glycolysis) were determined. Next, OCR and ECAR profiles in response to injection of glucose (25 mM), oligomycin (2 μM), FCCP (0.3 μM), and a combination of antimycin A (1 μM) and rotenone (1 μM) were evaluated. OCR and ECAR were measured using five 2 min cycles of mix and measurement following each injection. Normalization was performed by assessing total DNA using Quanti-iT PicoGreen (Life Technologies, Gaithersburg, MD, USA). Data analyses and calculations were performed as previously described in detail in (41).

Quantitative RT-PCR. RNA was extracted from cell lysates using RNeasy Mini Kit (Qiagen, Chatsworth, CA, USA) and was reverse-transcribed using QuantiTect Reverse Transcription Kit (Qiagen, Chatsworth, CA, USA). Gene expression levels were quantified by qPCR using QuantiFast SYBR Green PCR Kit (Qiagen, Chatsworth, CA, USA) and reactions were performed on a Viia™ 7 real time PCR system (Life Technologies, Gaithersburg, MD, USA). Gene-specific amplification was achieved using RT² qPCR Primer Assays (Qiagen, Chatsworth, CA, USA). Catalog numbers for each primer assays is presented in (Table 1). Relative gene expression was determined by normalizing the expression of each target gene to β-actin.

Immunoblot analysis. HIF-1α protein is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions (42). In order to avoid further HIF-1α degradation during whole cell lysates preparation, BRIN-BD11 cells were lysed directly with NuPAGE® LDS Sample Buffer (1X) (Thermo Fisher Scientific, San Jose, CA, USA) in the presence of protease and phosphatase inhibitors cocktail (Cell Signaling Technology, Danvers, MA, USA), similarly to (43). Protein extracts were separated by electrophoresis on 4–12% NuPAGE Bis-Tris Mini Gel and then transferred onto nitrocellulose membranes using iBlot transfer stacks (Life Technologies, Gaithersburg, MD, USA). Membranes were probed with various primary antibodies overnight at 4 °C. All washes and secondary antibody incubations were performed using the SNAP i.d. quick immunoblot vacuum system (Millipore, Billerica, MA, USA). Bands were developed using Clarity Western ECL substrate (Bio-Rad Laboratories, Hercules, CA, USA). Visualization and quantitative densitometry analysis were performed with Molecular Imager® Gel Doc™ XR System v5.2.1 (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis. Variables are described as mean ± S.E.M. Differences across groups were tested using ANOVA with a Tukey post hoc test when more than two experimental groups were analyzed and using Student's t-test when only two experimental groups were compared. Statistical significance was set at $P < 0.05$ (two-tailed). All analyses were performed using GraphPad Prism software v. 6.0.

RESULTS

GLP-1 Stimulates Glycolysis *via* Up-Regulation of Glycolytic Enzymes

Due to GLP-1 dependence on glucose for stimulation of insulin secretion (14), we investigated if GLP-1 signaling could modulate energy metabolism in β-cells. The rat insulin-secreting cell line BRIN-BD11 was stimulated for 18 hours with the GLP-1 analogue Exendin-4 and high (20 mM) glucose concentration. Media was removed and cells cultured for additional 24 hours in 20 mM of glucose, but in the absence of Exendin-4. The rationale for this approach was to investigate if GLP-1 signaling could cause metabolic reprograming that would persist even after removal of receptor stimulation. Glucose consumption, lactate production and total ATP content were determined after 24 hours incubation in the absence of Exendin-4, which followed previous 18 hours exposure to Exendin-4 (Fig. 1*A*-*C*). All three parameters were significantly enhanced in cells pre-conditioned with Exendin-4 relative to control untreated cells.

Next, we assessed glycolytic flux by measuring extracellular acidification rate (ECAR) using the XF^{e}96 flux analyzer (Seahorse Biosciences). For this, Exendin-4 treated and untreated cells were rested in base media (modified DMEM without glucose) for 1 hour, then ECAR was measured during sequential injections of 25 mM glucose and 2μM oligomycin (to inhibit mitochondrial ATP generation). Exendin-4 treated cells responded to both glucose and oligomycin injections by increasing their ECAR levels substantially higher than control cells (Fig. 1*D*). Basal glycolysis and glycolytic capacity can be calculated by subtraction of ECAR values obtained after glucose and oligomycin injections from basal ECAR levels respectively. In support of our glucose consumption

and lactate production data, ECAR measurements indicated that basal glycolysis (Fig. 1*E*) and glycolytic capacity (Fig. 1*F*) were both increased by Exendin-4 pre-conditioning. The fact that the observed effects on glycolysis are maintained after removal of receptor stimulation suggests that GLP-1 signaling caused metabolic reprograming and sustained gene expression changes in our β-cell model. To test for this, we assessed gene expression profiles of genes involved in the glycolytic pathway. Four of the six glycolytic genes investigated were significantly up-regulated by Exendin-4 pre-conditioning, including Lactate dehydrogenase A (*Ldha*), Aldolase A (*Aldoa*), Phosphofructokinase (*Pfkp*) and Glucose-6-phosphate isomerase (*Gpi*) (Fig. 1*G*).

GLP-1 Enhances Maximal Mitochondrial Respiration and Reserve Capacity

Extracellular flux analysis can be used to assess mitochondrial bioenergetics through measurement of oxygen consumption rate (OCR) in intact cells following the injection of specific mitochondrial inhibitors (23). We measured OCR in cells preconditioned (or not) with Exendin-4 after injection of glucose, oligomycin, carbonyl cyanide-*4*-(trifluoromethoxy) phenylhydrazone (FCCP) and a combination of rotenone and antimycin A (Fig. 2*A*). This strategy allowed us to estimate several mitochondrial parameters in response to GLP-1R stimulation. Maximal mitochondrial respiration and reserve capacity were strikingly enhanced by Exendin-4 preconditioning (Fig. 2*B* and 2*C*, respectively), whereas mitochondrial ATP turnover was slightly, but significantly increased (Fig. 2*D*). On the other hand, basal respiration (Fig. 2*E*), proton leak (Fig. 2*F*) and non-mitochondrial OCR (Fig. 2G*)* were unaffected. Interestingly, maximal respiration and reserve capacity are both estimated from OCR measurements after injection of FCCP, an uncoupling agent that elevates mitochondria to their maximum

electron transport capacity. Under these conditions, the production of glycolytic derived pyruvate becomes rate limiting for the Krebs cycle and, consequently, to mitochondrial electron transport. Our data suggest that GLP-1 does not affect mitochondrial bioenergetics directly, since basal respiration is unchanged after Exendin-4 treatment. The observed effects in maximal respiration and reserve capacity probably occur due to increased glycolysis and consequent augmented flux of pyruvate into the Krebs cycle.

PI3K/mTOR Axis Mediates GLP-1 Actions on Glucose Metabolism

GLP-1 can regulate cell size, function and proliferation in β-cells *via* PI3K/mTOR pathway (24; 25). Moreover, mTOR controls nutrient sensing and energy metabolism in various cell types (26). We sought to investigate if GLP-1 actions on glucose metabolism require mTOR activity. Co-incubation of Exendin-4 with either LY294002 or Torin-1, two specific inhibitors of PI3K and mTOR respectively, completely abrogated the Exendin-4 effect on glucose consumption (Fig. 3*A*). Realizing that the two inhibitors generated similar responses in terms of glucose consumption, we proceeded to extracellular flux analysis only with Torin 1. Determination of the ECAR profile (Fig. 3*B*) revealed that mTOR inhibition slightly impaired responsiveness to glucose, but entirely abrogated cellular ability to increase ECAR to compensate for oligomycin-induced blockade of mitochondrial ATP production. Most importantly, Torin 1 abolished the observed increase in ECAR in response to Exendin-4, confirming the idea that increased glycolysis induced by GLP-1 signaling is mediated by mTOR activity. Estimation of basal glycolysis (Fig. 3*C*) and glycolytic capacity (Fig.3*D*) indicated that, in the presence of Torin 1, Exendin-4 treated cells do not differ significantly from untreated controls. Next, we assessed the role of mTOR in mediating GLP-1 effects on

mitochondrial bioenergetics. For this, we generated OCR profiles of cells pre-conditioned with Torin 1 and Exendin-4 (Fig. 3*E*). mTOR inhibition not only impaired mitochondrial ATP turnover, but abolished the positive effect of Exendin-4 on this parameter (Fig. 3*F*). The effects of Exendin-4 on maximal mitochondrial respiration (Fig. 3*G*) and reserve capacity (Fig. 3*H*) were also blocked by inhibition of mTOR with Torin 1. Our data confirm the importance of mTOR activity in the regulation of β-cell energy metabolism and indicate that GLP-1 effects on glycolysis are mediated by the PI3K/mTOR pathway.

HIF-1α Accumulates Downstream of mTOR and Mediates GLP-1-Induced Metabolic Reprograming

GLP-1 has been shown to promote HIF-1α translation *via* induction of mTOR in insulin secreting INS-1 cells and mouse islets (15). We hypothesized that HIF-1α mediates GLP-1 effects on glycolysis in our β-cell model. Immunoblot analysis confirmed that Exendin-4 treatment induced HIF-1α protein levels similar to those achieved with Forskolin, a specific activator of adenylyl cyclase, in BRIN-BD11 cells (Fig. 4*A*). Gene expression analysis revealed no changes in *HIF-1α* mRNA, suggesting that GLP-1 induces HIF-1α protein by a post-transcriptional mechanism (Fig. 4*B*), consistent with previous studies (15). Next, we assessed how HIF-1α levels are modulated by the activity of PI3K/mTOR axis in this setting. Activity of mTOR was monitored directly, by assessment of serine 2448 (S2448) phosphorylation status. Inhibition of either PI3K or mTOR abolished accumulation of HIF-1α triggered by Exendin-4, suggesting that such effect occur downstream of mTOR (Fig. 4*C*). In addition, the two inhibitors caused HIF-1α protein to drop further bellow basal levels, indicating that mTOR pathway controls HIF-1α also in unstimulated conditions.

Moreover, phosphorylation of mTOR S2448 was enhanced by Exendin-4, confirming that GLP-1R signaling increases the activation state of mTOR, as previously demonstrated (15; 27; 28). In order to determine the role of HIF-1α on GLP-1 induced metabolic reprograming, we used siRNA transfection to knockdown HIF-1α expression. We successfully reduced HIF-1α mRNA levels by approximately 80% (Fig. 5*A*), which resulted in an approximate reduction in HIF-1α protein levels by 60% (Fig. 5*B*). We then sought to determine how HIF-1α knockdown influences the effects of Exendin-4 on glycolytic gene expression. We observed that HIF-1α knockdown caused a reduction in expression of all six glycolytic genes investigated, although only phosphoglycerate mutase 2 (*Pgam2*) was statistically significant (Fig. 5*C*). This was sufficient to cause a reduction in glucose consumption in response to Exendin-4 (Fig. 5*D*), indicating that HIF-1α is necessary for GLP-1 effects. To confirm the role of HIF-1α for GLP-1 mediated metabolic changes, we investigated islets from a β-cell-specific Hif-1α knockout mouse line (referred to herein as β-Hif1a-null mice). Islets isolated from β-Hif1a-null and Floxed control animals were pre-conditioned with Exendin-4 for 18h. Glucose consumption and total ATP content were determined after 24 hours in media containing 20 mM of glucose (Fig. 5*E* and 5*F*). Although this did not reach statistical significance (due to limited availability of islets for such experiments), β-Hif1a-null islets presented reduced glucose consumption and ATP levels in response to Exendin-4 compared to control islets. Our data confirms that GLP-1 signaling promotes mTOR dependent HIF-1α accumulation in β-cells. Moreover, it indicates that HIF-1α is necessary for the observed effects of GLP-1 on β-cell glucose metabolism.

DISCUSSION

Treatment of T2D patients with GLP-1 analogues results in various beneficial effects, including improvement of β-cell function and glucose homeostasis (4). Yet, the molecular mechanisms underlining GLP-1 mediated stimulation of insulin secretion are still not fully characterized. GLP-1-induced insulin secretion is generally believed to rely on the direct effects of cAMP signaling on ATP sensitive K^+ channels and Ca^{2+} handling (14). Such mechanisms occur very rapidly, within 5-15 min upon receptor binding, indicating that the actions are not mediated by altered gene expression, but likely by phosphorylation of protein components of the secretory machinery (13). However, several lines of evidence suggest that GLP-1 also induces long lasting effects on β-cells. For instance, GLP-1 analogues promote persistent improvement of β-cell function even four weeks after treatment interruption, both in rodents and humans (29; 30). Such observations are attributed to GLP-1-induced β-cell replication and neogenesis. In fact, GLP-1 exerts growth factor-like actions on β-cells, resulting in proliferation and survival (4). These involve transactivation of epidermal growth factor receptor (EGF-R) and insulin-like growth factor 1 receptor (IGF-1R), with subsequent activation of the PI3K/mTOR pathway (25; 31). This pathway plays a central role in regulating glycolysis and energy metabolism (32), which prompted us to investigate whether GLP-1 modifies these aspects of β-cell biology.

In this study, we provide evidence that GLP-1 induces a metabolic switch in β-cells. In our model, cells primed with the GLP-1 analogue Exendin-4 present higher glycolytic rates when returned to standard culture media in the absence of GLP-1R stimulation. This is concluded from evidence provided by glucose consumption and

lactate production measurements as well as ECAR and OCR profiles (Figs. 1 and 2). We believe that the effects on glucose metabolism occur *via* up-regulation of glycolytic enzymes, since the metabolic effects were accompanied by a significant increase in gene expression of critical glycolytic genes. Corroborating this hypothesis, HIF-1α, a transcriptional factor known to target the same genes, accumulates under GLP-1R stimulation, in our model (Fig. 4). In addition, HIF-1α depletion impacted glucose consumption and glycolytic gene expression in the same setting (Fig. 5). Furthermore, our data indicate that the mTOR pathway plays a crucial role in mediating GLP-1 effects on β-cell energy metabolism. This is concluded from experiments (Fig. 3), describing the effects of a specific mTOR inhibitor (Torin-1), which completely abrogated the metabolic actions of Exendin-4. Accordingly, HIF-1α accumulation is also prevented upon mTOR inhibition, reinforcing its importance for the observed metabolic switch. We and others have demonstrated that GLP-1 elevates β-cell ATP levels (33; 34), strengthening the hypothesis that the incretin hormone may promote β-cell function through regulation of energy metabolism, especially considering the central role that ATP generation and an increase in ATP/ADP ration plays during GSIS. We suspect that chronic metabolic/bioenergetic reprograming, observed in this study, could also mediate maintenance of β-cell function upon withdrawal of GLP-1 analogues, as documented in humans and rodents by others (29; 30).

Previously, GLP-1 has been reported not to influence β-cell energy metabolism (35). In that study, a short-term (90 min) stimulation of rodent islets with GLP-1 did not cause changes in glucose and lipid oxidation, although, the same study found a significant increase in glucose utilization, which perhaps has not been given sufficient

attention. We speculate that the increase in glucose utilization observed in (35) reflects a different mechanism, reported recently, in which GLP-1 induces rapid post-translational activation of glucokinase (36; 37). Our data support a mechanism dependent on sustained gene expression changes observed after a longer time period, which is consistent with previously identified delayed phase of gene expression changes mediated *via* HIF-1α (15).

In summary, this study describes a novel action of GLP-1 dependent signaling in β-cells, which stimulates metabolic/bioenergetic reprograming towards a higher glycolytic, and high ATP generating phenotype (Fig. 6). Such metabolic reprograming likely accounts for at least part of GLP-1 dependent insulin secretion. This provides an additional mechanism of action by which GLP-1 analogues improve glucose homeostasis in T2D patients, unveiling new aspects that could be useful for developing new therapeutic strategies.

AUTHOR CONTRIBUTIONS

R.C. and P.N. designed research; R.C., Y.C., J.R., V.C., K.K., L.E., and R.S. performed research; R.C., Y.C., V.C, and P.N. analyzed data; R.C, P.I.H.B., J.G., and P.N. wrote the paper. R.C and Y.C. had equal contribution to this study. R.C. and P.N. are the guarantors of this study and take full responsibility for the work as a whole, including the study design, access to data, and the decision to submit and publish the manuscript.

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Authors declare no potential conflicts of interest.

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FIGURE LEGENDS

Figure 1. Chronic GLP-1R signaling stimulates glycolysis in the rat insulin-secreting cell line BRIN-BD11. (A, B and C) Cells were incubated in the presence or absence of 50 nM Exendin-4 for 18 hours. Then, media was changed to RPMI containing 20 mM of glucose for additional 24 hours in the absence of Exendin-4. (A) Glucose consumption, (B) lactate production and (C) ATP content were assessed. (D) Following 18 hours of incubation in the presence or absence of 50 nM Exendin-4, BRIN-BD11 cells were subjected to extracellular flux analysis using Seahorse Bioscience XF^{e}96 Flux analyzer. ECAR was determined after sequential injection of 25 mM glucose and 2 μM oligomycin. (E and F) Glycolytic rate and capacity were calculated by subtracting the maximal values after glucose and oligomycin injections respectively, from basal ECAR levels. (G) Gene expression of glycolytic enzymes was assessed by qRT-PCR in BRIN-BD11 cells exposed to 50 nM Exendin-4 for 18 hours. Data represent mean ± SEM, n = 3; *P < 0.05; **P < 0.01; ***P < 0.001.

Figure 2. Maximal respiration and reserve capacity are increased in BRIN-BD11 cells by chronic exposure to Exendin-4. (A) Following 18 hours of incubation in the presence or absence of 50 nM Exendin-4, BRIN-BD11 cells were subjected to extracellular flux analysis using Seahorse Bioscience XF^{e}96 Flux analyzer. OCR was determined sequentially after injection of 25 mM glucose followed by a set of mitochondrial inhibitors (2 μM oligomycin, 0.3 μM FCCP and 1 μM each of rotenone and antimycin A) to generate a mitochondrial stress profile. (B-G) Mitochondrial parameters were calculated from the mitochondrial stress profile as described in the *Materials and*

Methods section. (B) Maximal respiration, (C) reserve capacity, (D) mitochondrial ATP turnover, (E) basal respiration, (F) proton leak, (G) non-mitochondrial OCR. Data represent mean ± SEM, n = 3; *P < 0.05; **P < 0.01.

Figure 3. Inhibition of the PI3K/mTOR axis abolishes the effects of GLP-1R signaling on glucose metabolism. (A) BRIN-BD11 cells were pre-incubated with either 1 μM Torin 1 or 50 μM LY294002 for 30 min, followed by 50 nM Exendin-4 for 18 hours. Then, media was changed to RPMI containing 20 mM of glucose for additional 24 hours in the absence of Exendin-4, then glucose consumption was determined. Data represent mean ± SEM, n = 4. (B-G) Cells were treated similarly to (A), but instead of the additional 24 hours incubation time, extracellular flux analysis was performed immediately following the 18 hours incubation with Exendin-4. (B) ECAR was determined after sequential injection of 25 mM glucose and 2 μM oligomycin. (C and D) Glycolytic rate and capacity were calculated by subtracting the maximal values after glucose and oligomycin injections respectively, from basal ECAR levels. (E) OCR was determined sequentially after injection of 25 mM glucose followed by a set of mitochondrial inhibitors (2 μM oligomycin, 0.3 μM FCCP and 1 μM each of rotenone and antimycin A) to generate a mitochondrial stress profile. (F) Mitochondrial ATP turnover, (G) maximal respiration and (H) reserve capacity were calculated. Data represent mean ± SEM, n = 3; *P < 0.05; **P < 0.01; ***P < 0.001.

Figure 4. GLP-1R signaling promotes HIF-1α protein expression downstream of PI3K/mTOR. (A) BRIN-BD11 cells were incubated in the presence or absence of 50 nM

Exendin-4 or 10 μM forskolin for 18 hours. Immunoblot shows that Exendin-4 treatment promotes protein expression of HIF-1α to a similar extent as forskolin treatment. (B) Gene expression analysis of HIF-1α mRNA by qRT-PCR, showing absence of statistic significant differences between Exendin-4 treated and untreated cells. Data represent mean ± SEM, n = 3; n.s. = non-significant. (C) BRIN-BD11 cells were pre-incubated with either 1 μM Torin 1 or 50 μM LY294002 for 30 min, followed by 50 nM Exendin-4 for 18 hours. Immunoblot shows Hif1α protein expression and phosphorylation levels of mTOR at serine 2448.

Figure 5. Increase in glucose metabolism induced by GLP-1R signaling is mediated by HIF-1α. (A-D) BRIN-BD11 cells were transfected with HIF-1α specific siRNAs (siHif1a), and subsequently exposed to 50 nM Exendin-4 for 18 hours. (A) Gene expression analysis of HIF-1α mRNA, siHif1a transfection caused a reduction of approximately 80% compared to non-targeting siRNA control (sicontrol). Data are mean ± SEM, n = 3; ***P < 0.001. (B) Immunoblot analysis of Hif1α protein expression, band densitometry analysis indicates a decrease of approximately 60% of HIF-1α protein expression in response to siHif1a transfection. (C) qRT-PCR analysis of glycolytic enzymes. (D) Glucose consumption assay, showing reduction in response to Exendin-4 in cells in which HIF-1α expression was silenced. Data are mean ± SEM, n = 4; *P < 0.05. (E and F) Pancreatic islets were isolated from β-cell specific HIF-1α knockout mice (β-Hif1a-null) or Floxed control counterparts. Islets were treated with 50 nM Exendin-4 similarly to previously described for BRIN-BD11 cells, then glucose consumption and ATP content were determined respectively. Data are mean ± SEM, n = 2-4.

Figure 6. GLP-1 receptor signaling promotes β-cell glucose metabolism *via* mTOR-dependent HIF-1α activation. Upon GLP-1 binding, the G-protein coupled GLP-1R receptor activates adenylyl cyclase, increasing intracellular levels of cAMP. Signaling mediated by cAMP promotes the mTOR pathway in β-cells, which, in turn, induces translational activation of HIF-1α. In the nucleus, HIF-1α drives transcriptional up-regulation of glycolytic genes. Increased pool of glycolytic enzymes in the cytosol allows for an elevated glycolytic flux and glycolytic capacity in the presence of high glucose, resulting in faster generation of ATP and enhanced GSIS.

Table 1. List of genes assessed by qRT-PCR and respective RT² qPCR Primer Assay catalog numbers.

Gene Symbol	Refseq #	Qiagen Catalog Number
Pklr	NM_012624	PPR50315
Pgam2	NM_017328	PPR44447
Gpi	NM_207592	PPR52501
Pfkp	NM_206847	PPR59714
Aldoa	NM_012495	PPR42582
Ldha	NM_017025	PPR56603

Figure 1.

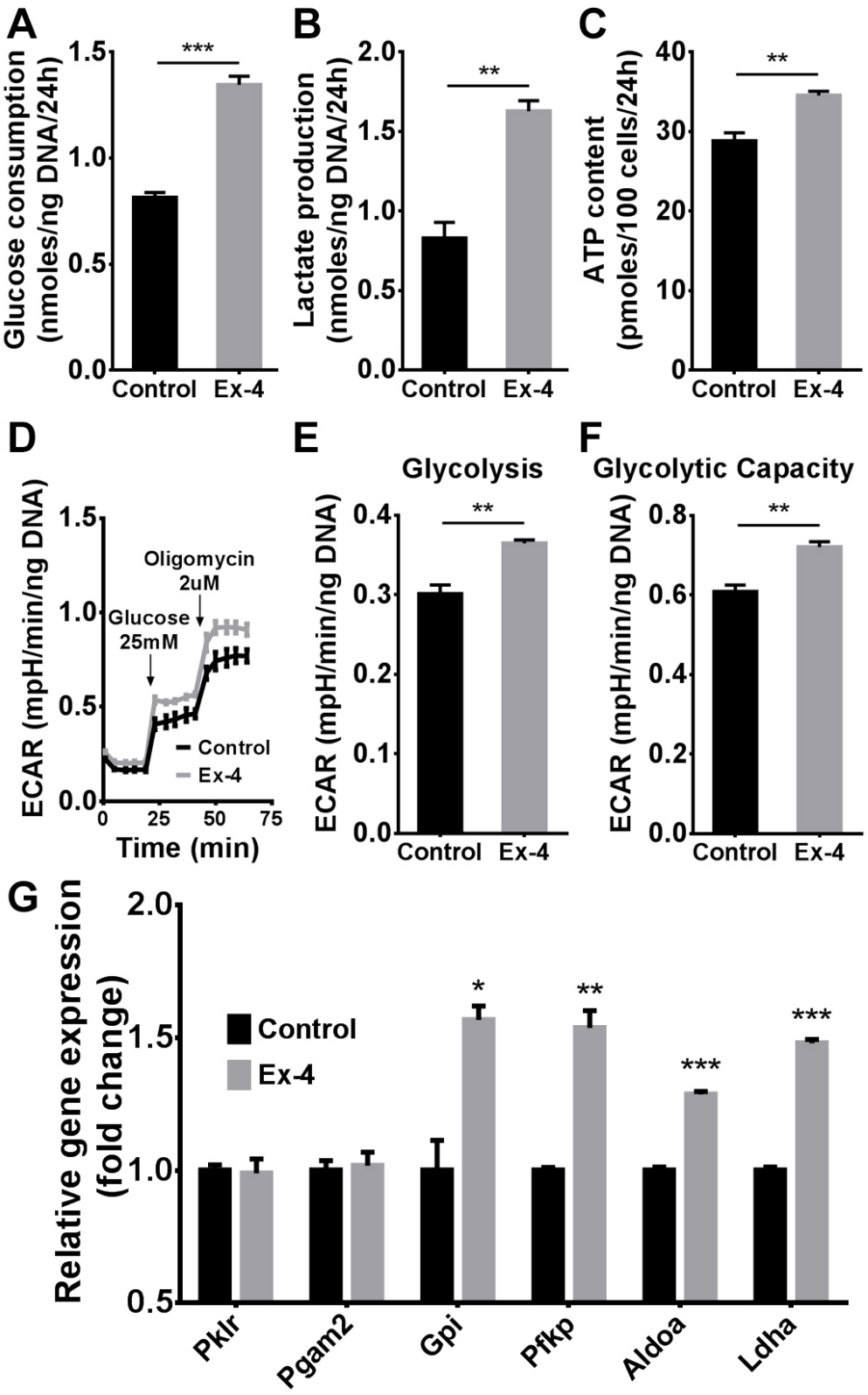

A
Glucose consumption (nmoles/ng DNA/24h)
1.5
1.0
0.5
0.0
***
Control
Ex-4
B
Lactate production (nmoles/ng DNA/24h)
2.0
1.5
1.0
0.5
0.0
**
Control
Ex-4
C
ATP content (pmoles/100 cells/24h)
40
30
20
10
0
**
Control
Ex-4
D
ECAR (mpH/min/ng DNA)
1.5
1.0
0.5
0.0
Oligomycin 2uM
Glucose 25mM
Control
Ex-4
0
25
50
75
Time (min)
E
Glycolysis
ECAR (mpH/min/ng DNA)
0.4
0.3
0.2
0.1
0.0
**
Control
Ex-4
F
Glycolytic Capacity
ECAR (mpH/min/ng DNA)
0.8
0.6
0.4
0.2
0.0
**
Control
Ex-4
G
Relative gene expression (fold change)
2.0
1.5
1.0
0.5
Control
Ex-4
*
**
***
***
Pklr
Pgam2
Gpi
Pfkp
Aldoa
Ldha

Figure 2.

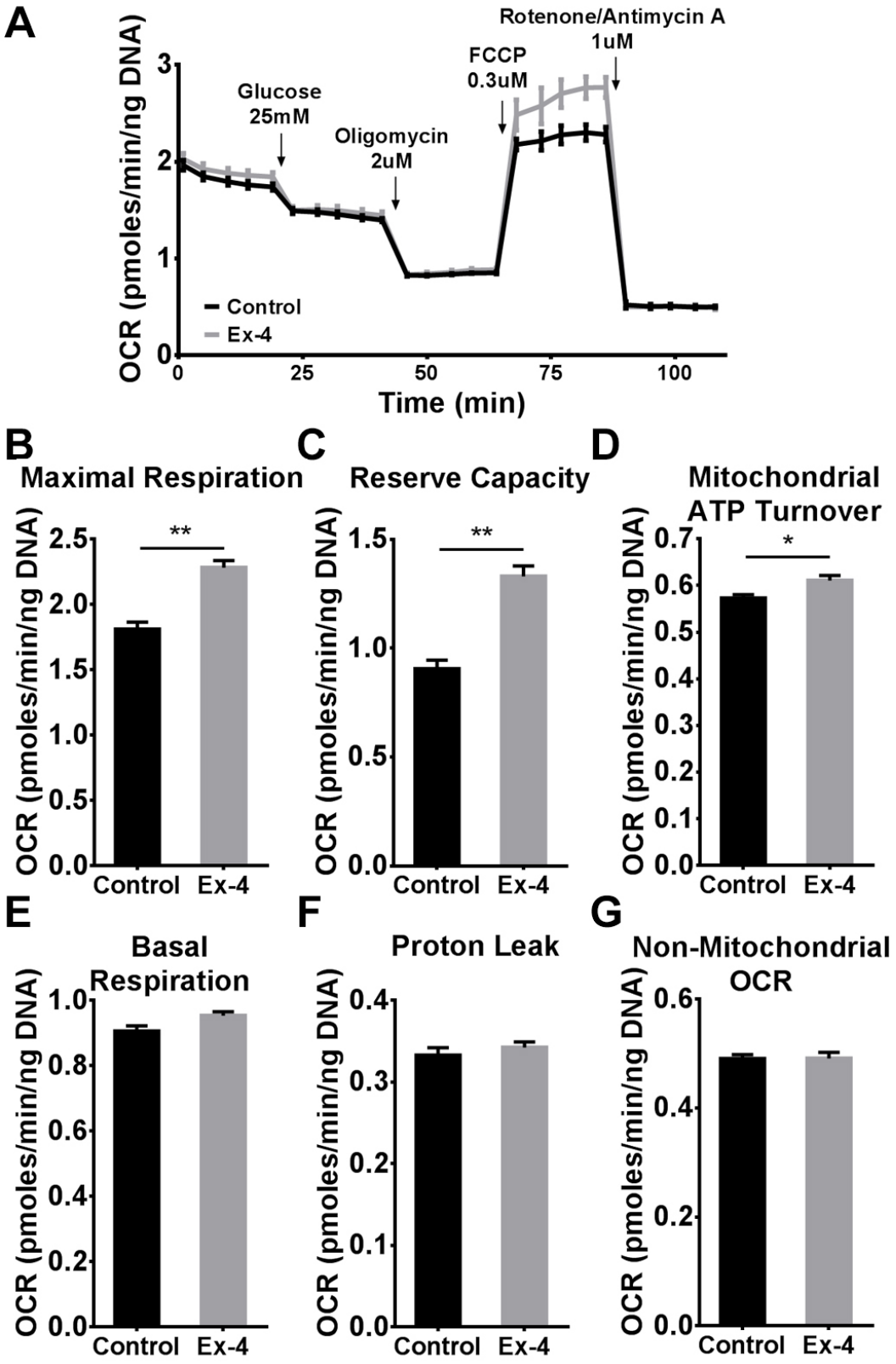

A
OCR (pmoles/min/ng DNA)
Time (min)
Glucose 25mM
Oligomycin 2uM
FCCP 0.3uM
Rotenone/Antimycin A 1uM
Control
Ex-4
B
Maximal Respiration
C
Reserve Capacity
D
Mitochondrial ATP Turnover
E
Basal Respiration
F
Proton Leak
G
Non-Mitochondrial OCR
Control
Ex-4

Figure 3.

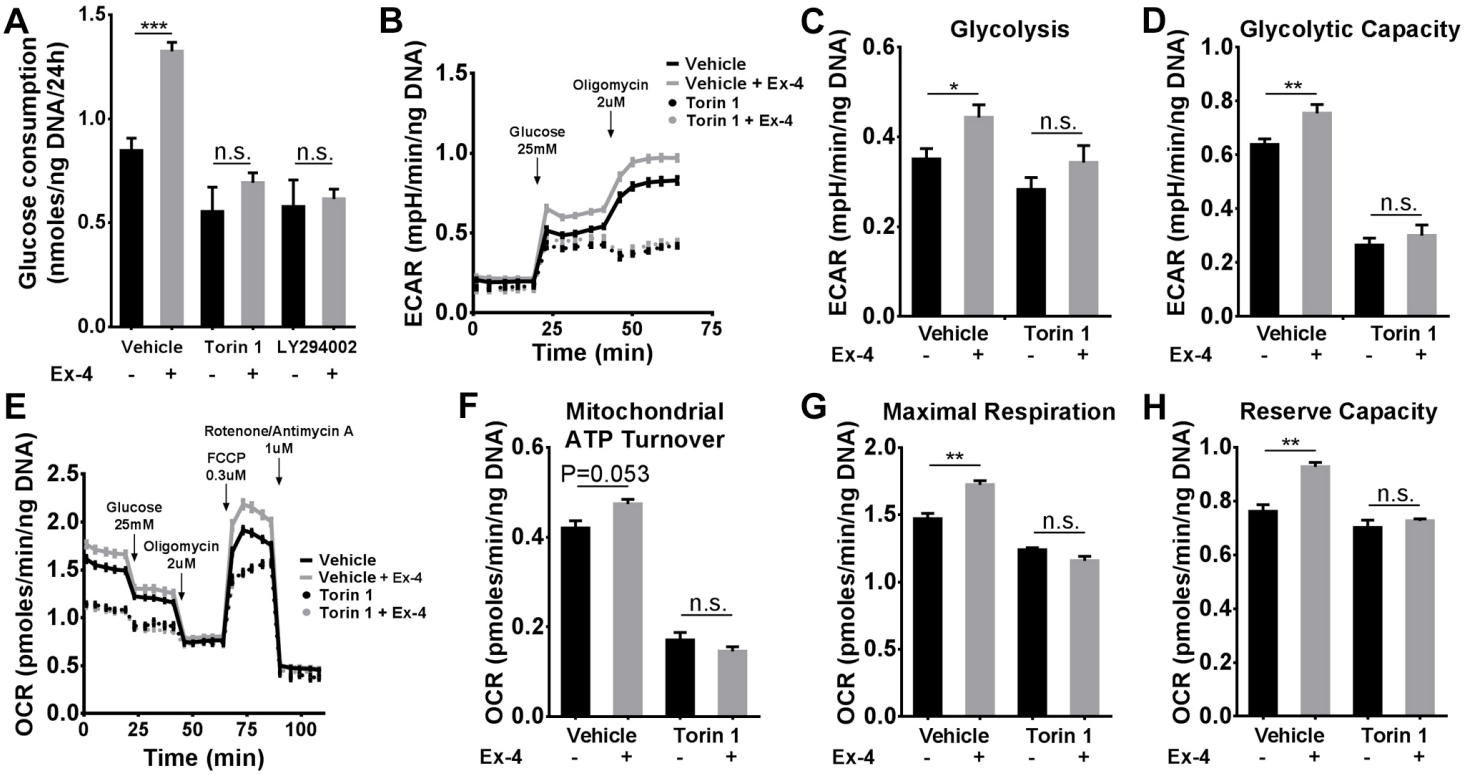

A
Glucose consumption (nmoles/ng DNA/24h)
***
n.s.
n.s.
Vehicle
Torin 1
LY294002
Ex-4
B
ECAR (mpH/min/ng DNA)
Glucose 25mM
Oligomycin 2uM
Vehicle
Vehicle + Ex-4
Torin 1
Torin 1 + Ex-4
Time (min)
C
Glycolysis
ECAR (mpH/min/ng DNA)
*
n.s.
Vehicle
Torin 1
Ex-4
D
Glycolytic Capacity
ECAR (mpH/min/ng DNA)
**
n.s.
Vehicle
Torin 1
Ex-4
E
OCR (pmoles/min/ng DNA)
Rotenone/Antimycin A 1uM
FCCP 0.3uM
Glucose 25mM
Oligomycin 2uM
Vehicle
Vehicle + Ex-4
Torin 1
Torin 1 + Ex-4
Time (min)
F
Mitochondrial ATP Turnover
P=0.053
n.s.
OCR (pmoles/min/ng DNA)
Vehicle
Torin 1
Ex-4
G
Maximal Respiration
**
n.s.
OCR (pmoles/min/ng DNA)
Vehicle
Torin 1
Ex-4
H
Reserve Capacity
**
n.s.
OCR (pmoles/min/ng DNA)
Vehicle
Torin 1
Ex-4

Figure 4.

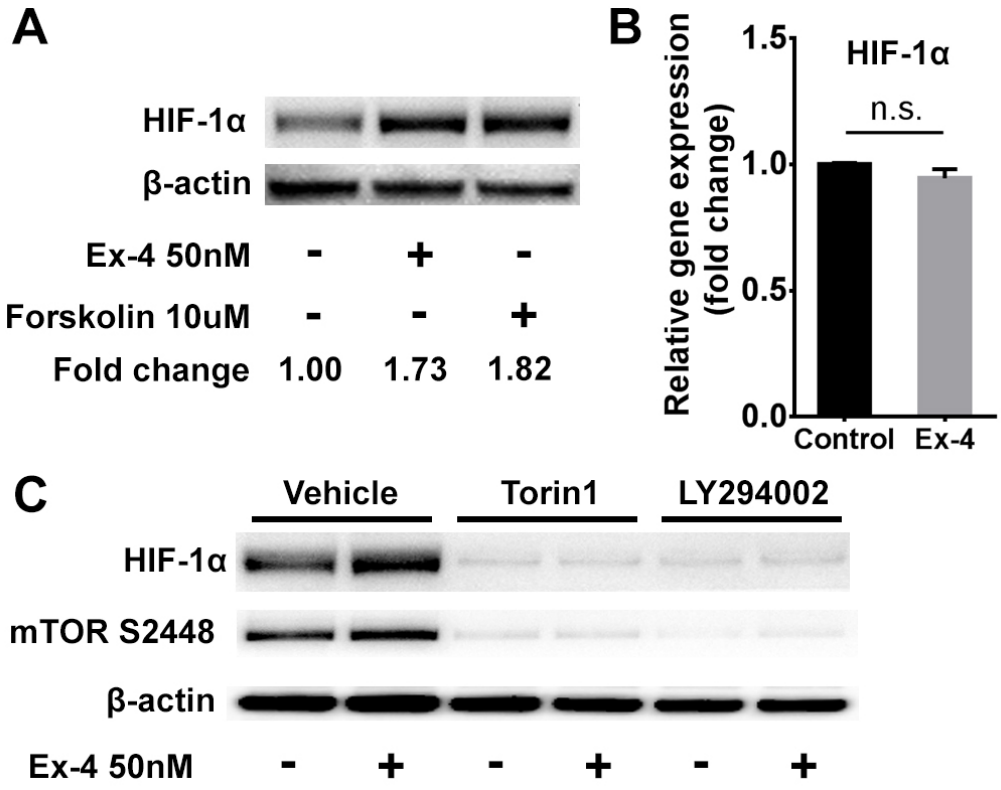

A
HIF-1α
β-actin
Ex-4 50nM - + -
Forskolin 10uM - - +
Fold change 1.00 1.73 1.82
B
Relative gene expression (fold change)
1.5
1.0
0.5
0.0
HIF-1α
n.s.
Control
Ex-4
C
Vehicle
Torin1
LY294002
HIF-1α
mTOR S2448
β-actin
Ex-4 50nM - + - + - +

Figure 5.

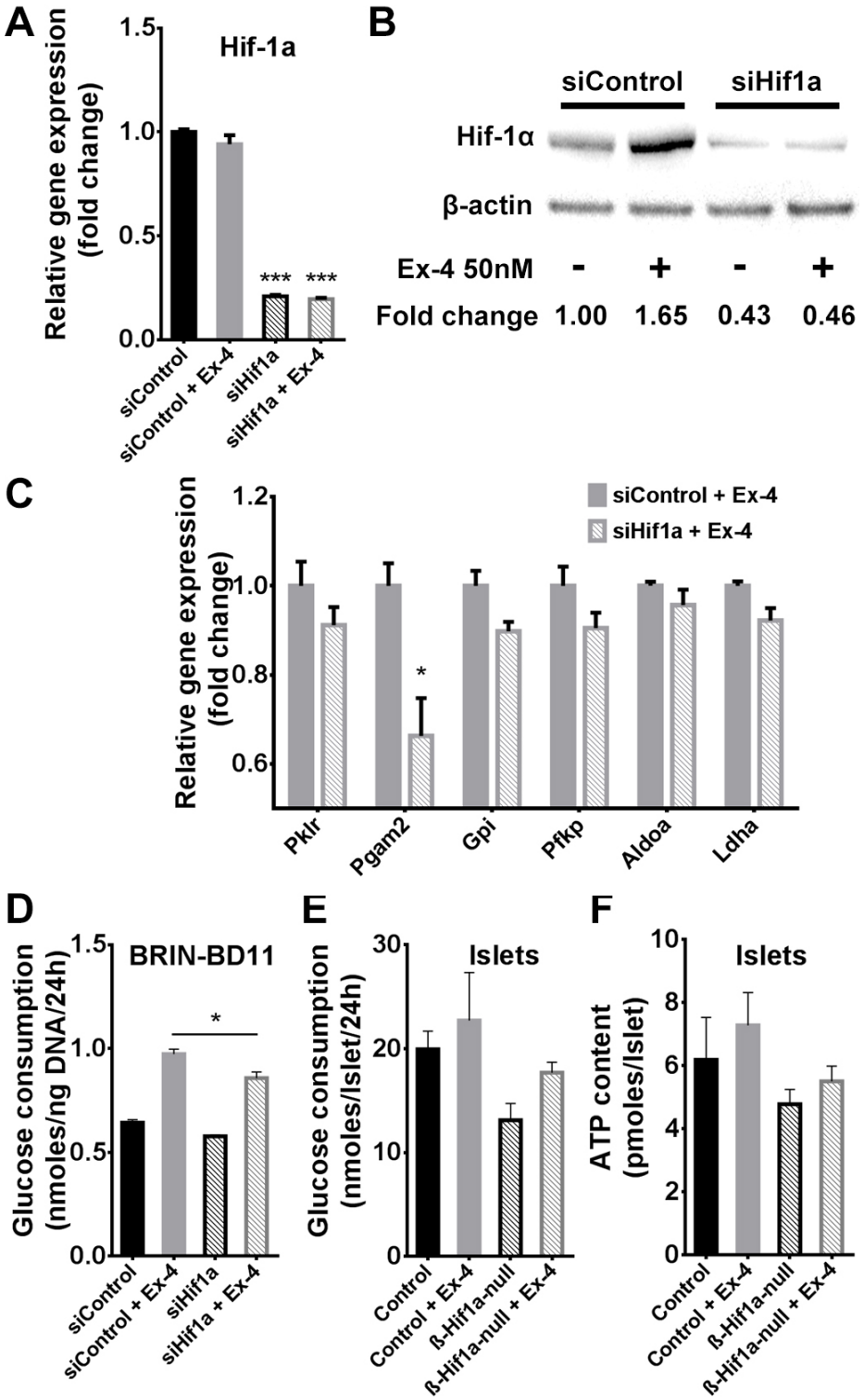
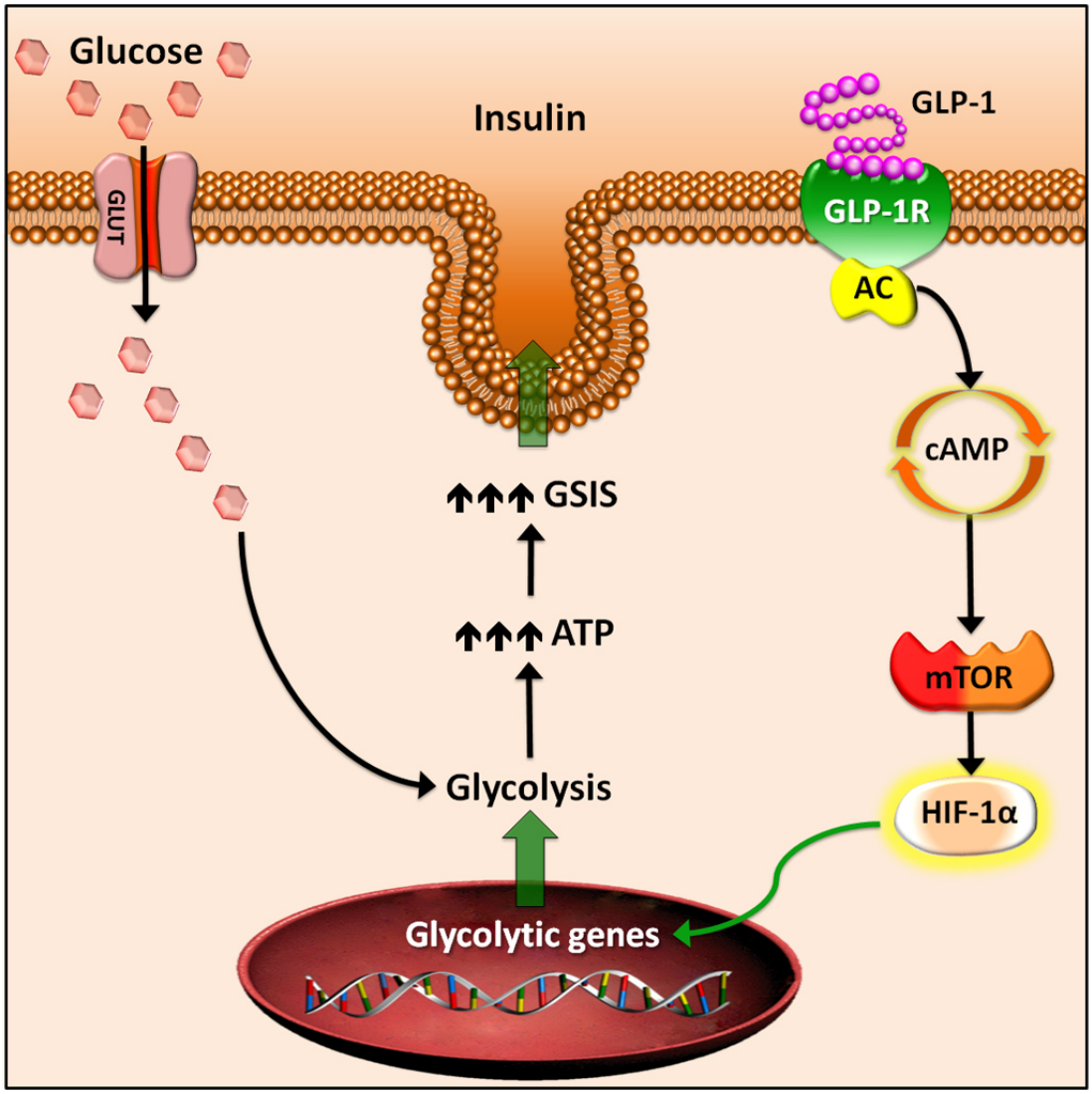

A
Relative gene expression (fold change)
Hif-1a
1.5
1.0
0.5
0.0
***
***
siControl
siControl + Ex-4
siHif1a
siHif1a + Ex-4
B
siControl
siHif1a
Hif-1α
β-actin
Ex-4 50nM - + - +
Fold change 1.00 1.65 0.43 0.46
C
Relative gene expression (fold change)
siControl + Ex-4
siHif1a + Ex-4
1.2
1.0
0.8
0.6
*
Pklr
Pgam2
Gpi
Pfkp
Aldoa
Ldha
D
Glucose consumption (nmoles/ng DNA/24h)
BRIN-BD11
1.5
1.0
0.5
0.0
*
siControl
siControl + Ex-4
siHif1a
siHif1a + Ex-4
E
Glucose consumption (nmoles/Islet/24h)
Islets
30
20
10
0
Control
Control + Ex-4
ß-Hif1a-null
ß-Hif1a-null + Ex-4
F
ATP content (pmoles/Islet)
Islets
10
8
6
4
2
0
Control
Control + Ex-4
ß-Hif1a-null
ß-Hif1a-null + Ex-4

Figure 6.


Glucose
Insulin
GLP-1
GLUT
GLP-1R
AC
cAMP
GSIS
ATP
mTOR
Glycolysis
HIF-1α
Glycolytic genes

4. CONCLUSÕES

Nossos resultados levaram à descoberta de efeitos benéficos do análogo do GLP-1, a Exendin-4, anteriormente desconhecidos. Entre eles, a proteção de ilhotas pancreáticas e do fígado contra efeitos deletérios conhecidos exercidos sobre esses tecidos pelo estresse inflamatório causado pela ME do doador.

Através da utilização de um modelo de ME em ratos, observou-se que a administração de Exendin-4 reduz significativamente as perdas de viabilidade e função em ilhotas isoladas. Análises de expressão gênica do tecido pancreático indicaram que o tratamento com Exendin-4 foi capaz de modular a expressão de genes relacionados a inflamação e estresse oxidativo. Já nas ilhotas, o tratamento com Exendin-4 foi associado com redução na expressão de genes relacionados ao estresse do retículo endoplasmático. Esses resultados sugerem que o efeito protetor exercido pela Exendin-4 se dá através da mitigação de processos fisiológicos sabidamente associados a disfunção e morte celular da célula β: inflamação, estresse oxidativo e estresse do retículo endoplasmático.

No fígado, o modelo de ME induziu apoptose de hepatócitos. Isso foi demonstrado pela ativação da Caspase-3, visualizada por imunoistoquímica e *western blot* de biópsias de fígado coletadas após seis horas do estabelecimento cirúrgico da ME. Isso se refletiu num aumento de marcadores de danos hepáticos circulantes, confirmando achados de estudos prévios. Interessantemente, animais tratados com Exendin-4 apresentaram níveis de ativação da Caspase-3 comparáveis aos animais controle, que não sofreram ME experimental. Isso também se traduziu em uma redução significativa nos níveis plasmáticos dos mesmos marcadores de danos hepáticos alterados em animais que sofreram a ME, mas não receberam a droga.

Digno de nota, esses achados têm o potencial para ser traduzidos para a prática clínica de transplantes de ilhotas e fígado no tratamento de pacientes com DM1 lábil e disfunção hepática, respectivamente. Contudo, tais resultados foram obtidos a partir de um modelo murino de ME e, portanto, devem ser estudados em ensaios clínicos randomizados para determinação de seu potencial em seres humanos, principalmente no que se refere aos desfechos pós-transplante. Após confirmação em ensaios clínicos, a inclusão de Exendin-4 ou outros análogos do GLP-1 no tratamento de doadores cadavéricos de órgãos poderá ajudar na melhoria da qualidade dos tecidos doados e, possivelmente, nos desfechos dos procedimentos de transplante de tais tecidos.

Adicionalmente, estudos realizados durante um período de 15 meses de doutorado sanduíche na Curtin University, Austrália, em colaboração com os professores Philip Newsholme e Paulo Ivo Homem de Bittencourt Junior, resultaram na elucidação de um mecanismo de transdução de sinal intracelular até o momento desconhecido, que liga a ativação do GLP-1R com o controle do metabolismo glicolítico na célula β. Nesse estudo, observou-se que o pré-condicionamento de células β em cultura na presença de estimulação do GLP-1R com Exendin-4 causa um aumento na taxa glicolítica das mesmas células quando retornadas ao meio de cultura padrão na ausência de estimulação. Isso foi demonstrado pela primeira vez neste estudo e indica que o GLP-1 induz a uma reprogramação metabólica até o momento desconhecida nas células β. Os dados sugerem que esse *switch* metabólico é mediado pela acumulação do fator de transcrição HIF-1α, levando a um aumento na expressão gênica de enzimas glicolíticas em um mecanismo dependente da via da mTOR. Acredita-se que esse mecanismo constitua parte importante do efeito incretina realizado pelo GLP-1 e também da ação de análogos do GLP-1 no

controle da homeostase glicêmica em pacientes com DM2. Novos aspectos da biologia da célula β e sua interação com o trato gastrointestinal através da sinalização mediada pelo GLP-1, portanto, foram elucidados neste estudo. Tais conhecimentos poderão ser úteis para o desenvolvimento de novas estratégias terapêuticas, capazes, por exemplo, de modular a função da célula β através do controle de sua atividade bioenergética, com impacto direto na secreção insulínica.

5. COLABORAÇÃO EM OUTROS TRABALHOS DURANTE O ANDAMENTO DO DOUTORADO

Além dos artigos que fazem parte da presente tese, ao longo do período de doutorado foram desenvolvidos, em colaboração, os seguintes manuscritos:

1. Younan Chen, **Rodrigo Carlessi**, Nikita Walsz, Vinicius Fernandes Cruzat, Kevin Keane, Philip Newsholme. "Pigment epithelium-derived factor (PEDF) regulates metabolism and insulin secretion from a clonal rat pancreatic beta cell line, BRIN-BD11". Submetido para publicação no periódico *Molecular and Cellular Endocrinology*.

2. Marjorie Piuco Buffon, Mariana Palazzo Carpena, Denise Alves Sortica, Andressa Santer, **Rodrigo Carlessi**, Bianca Marmontel de Souza, Maria Isabel Edelweiss, Milton Berger, Daisy Crispim, Luis Henrique Canani. "rs1888747 polymorphism in the FRMD3 gene, gene and protein expression: role in diabetic kidney disease". Submetido para publicação no periódico *Diabetology & Metabolic Syndrome.*

3. Keane, K. N., V. F. Cruzat, **R. Carlessi**, P. I. de Bittencourt, Jr. and P. Newsholme (2015). "Molecular events linking oxidative stress and inflammation to insulin resistance and beta-cell dysfunction". *Oxid Med Cell Longev* **2015**: 181643.

4. Rheinheimer, J., A. C. Bauer, S. P. Silveiro, A. A. Estivalet, A. P. Boucas, A. R. Rosa, B. M. de Souza, F. S. de Oliveira, L. A. Cruz, L. A. Brondani, M. J. Azevedo, N. E. Lemos, **R. Carlessi**, T. S. Assmann, J. L. Gross, C. B. Leitao and D. Crispim (2015). "Human pancreatic islet transplantation: an update and description of the establishment of a pancreatic islet isolation laboratory". *Arch Endocrinol Metab* **59**(2): 161-170.

5. Rheinheimer, J., P. K. Ziegelmann, **R. Carlessi**, L. R. Reck, A. C. Bauer, C. B. Leitao and D. Crispim (2014). "Different digestion enzymes used for human pancreatic islet isolation: a mixed treatment comparison (MTC) meta-analysis". *Islets* **6**(4).

6. Taís Silveira Assmann, Natália Emerim Lemos, Letícia de Almeida Brondani, **Rodrigo Carlessi**, Carmen Maldonado-Bernal, Miguel Cruz, Luis Henrique Canani and Daisy Crispim (2014). "Association between Asp299Gly and Thr399Ile polymorphisms in toll-like receptor 4 gene and type 2 diabetes mellitus: case-control study and meta-analysis". *J Diabetes Metab* **5**(9): 1000427.