UNIVERSIDADE ESTADUAL DO OESTE DO PARANÁ
CENTRO DE CIÊNCIAS BIOLÓGICAS E DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOCIÊNCIAS E SAÚDE – MESTRADO

CARINE MARMENTINI

PROLACTINA AUMENTA A EXPRESSÃO DE ENZIMAS ANTIOXIDANTES E ATIVA O RECEPTOR ATIVADO POR PROLIFERADOR DE PEROXISSOMA (PPAR) EM CÉLULAS INS-1E

CASCAVEL- PR
Fevereiro/2019

CARINE MARMENTINI

PROLACTINA AUMENTA A EXPRESSÃO DE ENZIMAS ANTIOXIDANTES E ATIVA O RECEPTOR ATIVADO POR PROLIFERADOR DE PEROXISSOMA (PPAR) EM CÉLULAS INS-1E

Dissertação apresentada ao Programa de Pós-Graduação em Biociências e Saúde – Mestrado, do Centro de Ciências Biológicas e da Saúde, da Universidade Estadual do Oeste do Paraná, como requisito parcial para a obtenção do título de Mestre em Biociências e Saúde.

Área de concentração: Biologia, processo saúde- doença e políticas de saúde

ORIENTADOR: Prof. Dr. Antonio Carlos Boschero

COORIENTADORA: Dra. Emerielle Cristine Vanzela

CASCAVEL- PR

Fevereiro/2019

Ficha de identificação da obra elaborada através do Formulário de Geração Automática do Sistema de Bibliotecas da Unioeste.

Marmentini, Carine
Prolactina aumenta a expressão de enzimas antioxidantes e ativa o receptor ativado por proliferador de peroxissoma (PPAR) em células INS-1E / Carine Marmentini; orientador(a), Antonio Carlos Boschero; coorientador(a), Emerielle Cristine Vanzela, coorientador(a)II, Sandra Lucinei Balbo, 2019.
104 f.

Dissertação (mestrado), Universidade Estadual do Oeste do Paraná, Campus de Cascavel, Centro de Ciências Biológicas e da Saúde, Programa de Pós-Graduação em Biociências e Saúde, 2019.

1. Células beta pancreáticas. 2. Prolactina. 3. Diabetes. 4. Receptor Ativado por Proliferadores de Peroxissoma. I. Boschero, Antonio Carlos. II. Vanzela, Emerielle Cristine . III. Balbo, Sandra Lucinei. IV. Título.

unioeste
Universidade Estadual do Oeste do Paraná
Campus de Cascavel CNPJ 78680337/0002-65
Rua Universitária, 2069 - Jardim Universitário - Cx. P. 000711 - CEP 85819-110
Fone:(45) 3220-3000 - Fax:(45) 3324-4566 - Cascavel - Paraná

PARANÁ
GOVERNO DO ESTADO

CARINE MARMENTINI

Prolactina aumenta a expressão de enzimas antioxidantes e ativa o receptor ativado por proliferadores de peroxissoma PPAR em células INS-1E

Dissertação apresentada ao Programa de Pós-Graduação em Biociências e Saúde em cumprimento parcial aos requisitos para obtenção do título de Mestra em Biociências e Saúde, área de concentração Biologia, Processo Saúde-doença e Políticas de Saúde, linha de pesquisa Fatores Que Influenciam A Morfofisiologia Orgânica, APROVADO(A) pela seguinte banca examinadora:

Antonio Carlos Boschero
Universidade Estadual do Oeste do Paraná (UNIOESTE)

Maria Lucia Bonfleur
Universidade Estadual do Oeste do Paraná - Campus de Cascavel (UNIOESTE)

Roberto Barbosa Bazotte
Universidade Estadual de Maringá (UEM)

Cascavel, 21 de fevereiro de 2019

DEDICATÓRIA

Dedico este trabalho à minha avó Ermelinda, que já se foi, mas estará sempre comigo em meu coração.

AGRADECIMENTOS

Aos meus pais, Elenir e Mauri, meus exemplos de dignidade e de dedicação, por nunca medirem esforços para que eu me dedicasse inteiramente aos estudos e sempre incentivarem-me a buscar novos desafios.

Ao meu irmão Maurício, que, apesar de mais novo, já me ensinou muito mais do que eu ensinei a ele. Obrigada por ser tão companheiro.

Ao meu melhor amigo e amor, Thiago, por tornar meus dias mais felizes e a vida mais leve, por ser meu porto seguro, apoiar minhas decisões e caminhar ao meu lado.

À Professora Sandra Balbo, por aceitar-me no Laboratório de Fisiologia Endócrina e Metabolismo da UNIOESTE desde meu primeiro ano da graduação. Obrigada pela confiança, pelo incentivo e por abrir-me tantas portas.

Ao meu orientador, Professor Antonio Boschero, pela oportunidade e todo o aprendizado que sua orientação proporcionou-me.

À Emerielle, por ter me guiado durante todo o mestrado, contribuindo diretamente tanto na fase experimental quanto na escrita do trabalho.

À Professora Leticia Roma, pela experiência incrível que me proporcionou ao disponibilizar seu laboratório na Alemanha para a realização de um estágio. Agradeço também pela solicitude e amizade.

Aos professores da banca de qualificação e defesa: Sabrina Grassiolli, Maria Lúcia Frizon, Roberto Bazotte e Maria Lúcia Bonfleur, por aceitarem o convite e pelas contribuições para o aprimoramento do trabalho.

Às amizades que ganhei durante a graduação e mantenho até hoje: Ana, Luiza, Mariana e Mayara, por toda a cumplicidade e história que vivemos juntas.

Às Gabis e à Jakeline, com quem sempre pude contar, em qualquer momento e para qualquer situação. Agradeço também pela companhia, pelas risadas e pelas ótimas conversas.

A todos os membros do Laboratório de Pâncreas Endócrino e Metabolismo da UNICAMP, pelo auxílio prestado durante o desenvolvimento dos experimentos e pelo enorme aprendizado que a convivência diária proporcionou-me. Em especial, à Cristiane, à Israelle, ao Jean, à Maressa, à Mirian, à Sandra, ao Thiago Reis, ao Thiago Rentz e ao José Maria, pela amizade construída durante o período em que

estive lá.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), à Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) e ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), pelo auxílio financeiro para o desenvolvimento deste trabalho.

À todos os professores, os quais em algum momento fizeram parte da minha formação.

À todos que por ventura não foram citados, mas que contribuíram direta ou indiretamente para o meu desenvolvimento pessoal e profissional.

RESUMO

MARMENTINI, C. **Prolactina aumenta a expressão de enzimas antioxidantes e ativa o receptor ativado por proliferador de peroxissoma (ppar) em células INS-1E.** 101 páginas. Dissertação (Mestrado). Programa de Pós-Graduação em Biociências e Saúde, Centro de Ciências Biológicas e da Saúde, Campus Cascavel, UNIOESTE, 2019.

O diabetes mellitus (tipos 1 e 2) é caracterizado pela morte de células beta pancreáticas, a qual é induzida, entre outros fatores, pelo estresse oxidativo (EO). Sabe-se que a prolactina (PRL) melhora a sobrevivência de células beta frente a essa condição; entretanto, o mecanismo pelo qual isso ocorre ainda é desconhecido. Uma vez que a PRL modula a expressão gênica de enzimas antioxidantes em diferentes tipos celulares, nesta pesquisa investigamos se a PRL protege as células beta pancreáticas do estresse oxidativo por aumentar a produção de enzimas antioxidantes e quais vias de sinalização estariam envolvidas nesse efeito. Para isso, utilizamos células de insulinoma de rato da linhagem INS-1E. As células foram pré-tratadas por 24h com veículo (0,3 mmol/L de $NaHCO_3$ + 0,3% de BSA) ou PRL (0,5 µg/mL), seguido da indução do estresse oxidativo com peróxido de hidrogênio (H_2O_2) para avaliação da viabilidade celular, produção mitocondrial de espécies reativas de oxigênio (EROs) e conteúdo proteico das enzimas antioxidantes catalase (CAT), glutationa peroxidase 1 (GPx1), superóxido dismutase 1 (SOD1) e superóxido dismutase 2 (SOD2). O conteúdo proteico dessas enzimas também foi avaliado no experimento de *time-course*, no qual as células foram tratadas com PRL (0,5 µg/mL) em diferentes tempos. Fisiologicamente, durante a prenhes/gravidez ocorre acentuada secreção hipofisária de PRL. Por isso, com o objetivo de elencarmos possíveis vias de sinalização a serem investigadas, nosso próximo passo foi realizar análises de bioinformática com os genes *upregulated* em ilhotas de ratas prenhas, identificados previamente por nosso grupo de pesquisa a partir de *macroarray*. Os achados dessas análises levaram-nos à realização do ensaio de promotor repórter utilizando diferentes concentrações de PRL por 24h. Por fim, para testar se os efeitos da PRL são mediados pela via de sinalização sugerida pelas análises de bioinformática, avaliamos novamente a viabilidade celular na presença de siRNA para *knockdown* do gene alvo. As análises estatísticas foram feitas por teste t ou ANOVA seguida do pós-teste de Bonferroni. A PRL aumentou a sobrevivência das células beta pancreáticas expostas a H_2O_2, assim como o conteúdo proteico da SOD2 e da CAT. Também observamos a diminuição da concentração mitocondrial de H_2O_2 em células pré-tratadas com o hormônio. As análises de bioinformática revelaram enriquecimento da via do receptor ativado por proliferadores de peroxissoma (PPAR), o qual está envolvido na transcrição de enzimas antioxidantes. Com o ensaio de promotor repórter observamos que PRL ativa PPAR. Dentre as isoformas de PPAR existentes, avaliamos se PRL seria dependente de PPARγ para evitar a morte celular causada pelo EO, uma vez que a expressão de PPARγ é aumentada pela PRL em linhagem celular de pré-adipócitos e no tecido adiposo de ratos. No entanto, o *knockdown* desse fator de transcrição não alterou a eficiência da PRL em proteger as células beta da morte causada pelo H_2O_2. Concluímos que a PRL aumenta a expressão de enzimas antioxidantes e essa ação parece ser independente de PPARγ. Investigações futuras são necessárias para identificar a via de sinalização que medeia esse efeito.

Palavras-chaves: células beta pancreáticas; estresse oxidativo; morte celular; prolactina; diabetes; PPAR

ABSTRACT

MARMENTINI, C. **Prolactin increases the expression of antioxidant enzymes and activates peroxisome proliferator-activated receptor (PPAR) in INS-1E cells.** 101 páginas. Dissertação (Mestrado). Programa de Pós-Graduação em Biociências e Saúde, Centro de Ciências Biológicas e da Saúde, Campus Cascavel, UNIOESTE, 2019.

Diabetes mellitus (types 1 and 2) is characterized by pancreatic beta cells death induced, among other factors, by oxidative stress (OE). It is known that prolactin (PRL) improves the survival of beta cells against this condition, however the mechanism by which this occurs is still unknown. Since PRL modulates the gene expression of antioxidant enzymes in different cell types, our objective was to investigate whether PRL protects pancreatic beta cells from oxidative stress by increasing the production of antioxidant enzymes and what signaling pathways would be involved in this effect. For this, we used beta cell line from rats insulinoma, INS-1E. Cells were pretreated for 24 h with vehicle (0.3 mmol/L $NaHCO_3$ + 0.3% BSA) or PRL (0.5 µg/mL), followed by induction of oxidative stress with hydrogen peroxide (H_2O_2) for evaluation of cell viability, mitochondrial production of reactive oxygen species (ROS) and protein content of antioxidant enzymes catalase (CAT), glutathione peroxidase 1 (GPx1), superoxide dismutase 1 (SOD1) and superoxide dismutase 2 (SOD2). The protein content of these enzymes was also evaluated in the time-course experiment, in which the cells were treated with PRL (0.5 µg/mL) at different times. Physiologically, during pregnancy there is marked pituitary secretion of PRL. Therefore, in order to indicate possible signaling pathways to be investigated, our next step was to perform bioinformatics analyzes with the genes upregulated in islets of pregnant rats previously identified by our research group from macroarray. The findings of these analyzes led us to carry out the reporter promoter assay using different concentrations of PRL for 24h. Finally, to test whether the effects of PRL are mediated by the signaling pathway suggested by bioinformatics analyzes, we again evaluate cell viability in the presence of siRNA for target gene knockdown. Statistical analyzes were performed by t-test or ANOVA followed by the Bonferroni posttest. PRL increased the survival of pancreatic beta cells exposed to H_2O_2, as well as the protein content of SOD2 and CAT. We also observed decrease in mitochondrial H_2O_2 concentration in cells pretreated with the hormone. Bioinformatics analyzes revealed enrichment of the peroxisome proliferator-activated receptor (PPAR) pathway, which is involved in the transcription of antioxidant enzymes. Using the reporter promoter assay we observed that PRL activates PPAR. Among the existing PPAR isoforms, we evaluated whether PRL would be dependent on PPARγ to prevent cell death caused by EO, since PPARγ expression is increased by PRL in preadipocyte cell line and in adipose tissue of rats. However, the knockdown of this transcription factor did not alter the efficiency of PRL in protecting beta cells from death caused by H_2O_2. We conclude that PRL increases the expression of antioxidant enzymes and this action seems to be independent of PPARγ. Future investigations are needed to identify the signaling pathway that mediates this effect.

Keywords: pancreatic beta cells; cell death; oxidative stress; prolactin; diabetes; PPAR

LISTA DE ILUSTRAÇÕES

LISTA DE TABELAS

LISTA DE ABREVIATURAS

ADP: adenosina difosfato
ARE: elementos de resposta antioxidante
ATP: adenosina trifosfato
cAMP: monofosfato cíclico de adenosina
CAT: catalase
CCK: colecistocinina
DAG: diacilglicerol
DM: diabetes mellitus
DM1: diabetes mellitus do tipo 1
DM2: diabetes mellitus do tipo 2
DNA: ácido desoxirribonucleico
EO: estresse oxidativo
EROs: espécies reativas de oxigênio
FAD: flavina adenina dinucleotídeo
GLUT: Transportador de glicose
GLP-1: peptídeo semelhante ao glucagon 1
GLP1R: receptor de GLP-1
GPx: glutationa peroxidase
IKKβ: I-kappa-B-quinase beta
IFN-γ: Interferon gama
IL-1β: Interleucina 1 beta
IP_3: inositol trifosfato
IR: receptor de insulina
IRS: substrato do receptor de insulina
JAK2: janus quinase 2
JNK: c-jun-N-terminal quinase
LAFEM: Laboratório de Fisiologia Endócrina e Metabolismo
LAPEM: Laboratório de Pâncreas Endócrino e Metabolismo
MAPK: proteíno-quinase ativada por mitógenos
mRNA: RNA (ácido ribonucleico) mensageiro
NAD: nicotinamida adenina dinucleotídeo
NF-Kb: fator nuclear kappa B
PCK: proteinoquinase C
PDX-1: fator promotor de insulina 1
PI3K: fosfatidilinositol 3-quinase
PKA: proteinoquinase A
PLC: fosfolipase C
PPARs: receptores ativados por proliferadores de peroxissoma
PPRE: elementos responsivos aos proliferadores de peroxissoma
PRL: prolactina
Prlr: receptor PRL
RXR: receptor x retinoide
SOD: superóxido dismutase
STAT: transdutor de sinal e ativador de transcrição
TNF-α: fator de necrose tumoral alfa
TSP-1: Trombospondina 1
UCP: proteínas de desacoplamento

SUMÁRIO

1. INTRODUÇÃO

O diabetes mellitus (DM) é uma doença crônica multifatorial, caracterizada por elevada concentração sanguínea de glicose, denominada “hiperglicemia”. Essa hiperglicemia pode ser decorrente da produção insuficiente de insulina ou da resistência na sua ação (WHO, 2018).

Nas últimas décadas, a prevalência do DM aumentou de forma muito expressiva, tornando essa doença uma das maiores preocupações globais em saúde do século XXI (IDF, 2015). Atualmente, cerca de 422 milhões de adultos em todo o mundo têm DM e 1,6 milhões de mortes por ano vêm sendo atribuídas às suas complicações (WHO, 2018). No Brasil, os dados não são menos alarmantes. De acordo com a Federação Internacional do Diabetes (IDF, 2015), em âmbito mundial, o país ocupa o 3º lugar no número de crianças de 0 a 14 anos com diabetes do tipo 1 (DM1) e o 4º lugar no número de adultos com diabetes do tipo 2 (DM2).

Dentre os tipos de DM, o DM1 e o DM2 são os mais recorrentes. O DM1 representa de 5 a 10% dos casos (DANEMAN, 2006; SBD, 2018), e é caracterizado pela destruição seletiva das células beta do pâncreas pelo sistema imune, o que resulta na deficiência de insulina (THOMAS et al., 2009). A causa exata do DM1 é desconhecida, mas o que se sabe é que advém de uma complexa interação entre os fatores genéticos e ambientais (WHO, 2016). Já o DM2, diagnosticado em 90 a 95% dos casos, pode ser decorrente da resistência à insulina e/ou da deficiência na sua secreção (SALSALI; NATHAN, 2006), e está associado principalmente ao sedentarismo, à dieta rica em carboidratos e aos lipídeos e envelhecimento. Pode ocorrer em qualquer idade, mas geralmente é diagnosticado após os 40 anos (SBD, 2018).

O estresse oxidativo (EO), que é o aumento na relação entre a produção de espécies moleculares altamente reativas e as defesas antioxidantes (SIES; JONES, 2007), contribui para a ativação do ataque autoimune contra as células beta pancreáticas (CERIELLO, 2000; LICHTENBERG; PINCHUK, 2015; NEWSHOLME et al., 2007) e prejudica a via de sinalização da insulina, condições estritamente

associadas ao DM1 e DM2 (MATOUGH et al., 2012).

Dentre as espécies reativas, as de oxigênio (EROs) estão presente em maior quantidade nas células e são representadas principalmente pela hidroxila (OH^-), pelo superóxido (O_2^-), pelo hidroperóxido (HO_2^-) e pelo peróxido de hidrogênio (H_2O_2). Em concentrações fisiológicas, elas são necessárias para a expressão gênica, para a supressão de células tumorais, para a ativação de fatores de transcrição nuclear e de crescimento celular, além de atuarem como mecanismo de defesa contra infecções (LEE; KOO; MIN, 2004)**.** Todavia, em excesso, esses compostos tornam-se nocivos por conta da sua ação deletéria sobre proteínas, lipídeos e DNA. Nas proteínas, as EROs provocam mudanças estruturais a partir da modificação dos resíduos de aminoácidos, resultando na diminuição da atividade proteica e na maior suscetibilidade à desnaturação e à hidrólise. Nos lipídeos, o processo mais frequente é o de oxidação, que diminui a fluidez das membranas e a seletividade no transporte iônico e promove a formação de produtos tóxicos para as células (GIROTTI, 1998; LEE; KOO; MIN, 2004). Quanto ao DNA, podem ocorrer mudanças na sequência das bases nitrogenadas, alterando a expressão gênica e favorecendo a patogenia de doenças crônicas (SINGH et al., 2009).

Para manter a concentração apropriada de EROs e evitar o EO, as células têm sistemas de defesa antioxidante que abrangem enzimas como a catalase (CAT), a glutationa peroxidase (GPx) e a superóxido dismutase (SOD) (VALKO et al., 2007). A SOD é responsável pela dismutação do O_2^-, formando o H_2O_2. O H_2O_2 pode ser metabolizado pela CAT ou pela GPx. No primeiro caso, ocorre a produção de uma molécula de H_2O e O_2 e, no segundo, duas moléculas de H_2O (LEE; KOO; MIN, 2004).

A prolactina (PRL), hormônio proteico secretado predominantemente pela hipófise anterior (FRIESEN; GUYDA; HARDY, 1970; IGNACAK et al., 2012), tem efeitos sobre o pâncreas como o aumento da secreção de insulina estimulada pela glicose e da expressão de transportador de glicose tipo 2 (GLUT2) em ilhotas de ratos neonatos (BOSCHERO et al., 1992; CREPALDI; CARNEIRO; BOSCHERO, 1997); acréscimo da massa de ilhotas pancreáticas e da secreção de insulina em ratas prenhas (BRELJE et al., 1993; SORENSON; BRELJE, 1997; AMARAL et al., 2004); aumento da vascularização em ilhotas de ratos transplantadas (JOHANSSON et al., 2009); e melhora da viabilidade de células beta humanas

expostas à citocinas pró-inflamatórias e H_2O_2 (YAMAMOTO et al., 2010). Recentemente, foram descritas vias envolvidas na proteção das células beta pela PRL frente ao insulto causado por citocinas pró-inflamatórias (NARDELLI et al., 2018). Entretanto, ainda são desconhecidos os mecanismos pelos quais esse hormônio protege as células beta do estresse oxidativo.

Nesse sentido, estudos já demostraram que a PRL aumenta a expressão gênica da SOD em ilhotas pancreáticas de ratas (BORDIN et al., 2004) e em linhagem de fibroblastos (ADACHI et al., 2004). Além disso, em células da retina de camundongos submetidos ao estresse oxidativo, a ausência do receptor de PRL diminuiu o mRNA de CAT (MELÉNDEZ GARCÍA et al., 2016). Dessa forma, investigamos se a proteção exercida pela PRL sobre as células beta pancreáticas contra o estresse oxidativo deve-se ao aumento da produção das enzimas antioxidantes e qual via de sinalização estaria envolvida nesse efeito.

2. OBJETIVOS

2.1 Objetivo Geral

Investigar mecanismos envolvidos na proteção das células beta pancreáticas pela PRL frente ao estresse oxidativo.

2.2. Objetivos específicos

Em linhagem de células beta pancreáticas (INS-1E), verificar se a PRL:

- Previne a morte celular induzida pelo H_2O_2;
- Aumenta a expressão de enzimas antioxidantes;
- Diminui a produção de EROs;
- Modula vias de sinalização envolvidas na expressão de enzimas antioxidantes.

3. REVISÃO DE LITERATURA

3.1 Produção, secreção e ação da insulina

O pâncreas é uma glândula dividida anatomicamente em três regiões: cabeça (proximal), corpo e cauda (distal). A primeira encontra-se em contato com o duodeno, enquanto a última com o hilo esplênico e flexura cólica esquerda. O pâncreas é constituído por uma porção exócrina e outra endócrina. A exócrina é formada pelos ácinos, responsáveis pela secreção de suco pancreático no duodeno, favorecendo a digestão (DOLENSEK; RUPNIK; STOZER, 2015). A endócrina é formada pelas ilhotas de Langerhans, descritas pela primeira vez em 1869, por Paul Langerhans (YAGIHASHI, 2015).

Para a manutenção de concentrações adequadas de nutrientes no sangue, as ilhotas pancreáticas secretam hormônios que agem em diversos tecidos, promovendo a captação de glicose, de aminoácidos e de ácidos graxos quando há excesso desses substratos, como é o caso da insulina, ou a liberação deles quando estão em concentrações insuficientes, ação que é induzida pelo glucagon, por exemplo. (HARDIE, 2012). Um pâncreas adulto normal contém cerca de 1 milhão de ilhotas, o que constitui até 2% da massa pancreática (ELIASCHEWITZ et al., 2009). As ilhotas de Langerhans de humanos são formadas principalmente pelas células beta (54%), pelas células alfa (34%) e pelas células delta (10%). Essas células secretam insulina, glucagon e somatostatina, respectivamente (BRISSOVA et al., 2005).

A fim de compreender acerca da fisiopatologia do diabetes, primeiramente é necessário entender como ocorre a produção e a secreção de insulina em condições fisiológicas normais, como fizemos nos parágrafos seguintes.

A insulina é um hormônio anabólico e, entre outras ações, é responsável pela captação de glicose principalmente pelas células musculares e adiposas, ou seja, pela redução da glicemia. Sua síntese é estimulada por nutrientes tais como glicose, aminoácidos e lipídeos (HABER et al., 2001), e tem início com a tradução do mRNA da insulina pelos ribossomos do retículo endoplasmático, formando um

pré-pró-hormônio da insulina. Essa molécula tem em torno de 11,5 kDa e é constituída por quatro domínios: peptídeo C, cadeias A e B e um peptídeo sinalizador. (DODSON; STEINER, 1998; SKELIN; RUPNIK; CENCIC, 2010). Nessa mesma organela, a pré-pró-insulina sofre clivagem de seu peptídeo sinalizador, dando origem à pró-insulina. A pró-insulina, com 9,0 kDa, é transportada até o complexo de Golgi, onde é armazenada em vesículas. Após a clivagem do peptídeo C dentro das vesículas, a insulina com 5,8 kDa, composta pelas cadeias A e B, está pronta para ser secretada (DODSON; STEINER, 1998).

A glicose é o principal estímulo para a secreção da insulina e a sua concentração no interstício varia em paralelo à concentração do substrato no sangue. A entrada de glicose nas células beta é facilitada pelo transportador de glicose (GLUT). Dentro da célula, a glicose é metabolizada, elevando a proporção de ATP/ADP, o que fecha os canais de K+ sensíveis a ATP e provoca a despolarização da membrana da célula beta. A despolarização promove a abertura dos canais de Ca^{2+} controlados por voltagem e a entrada desse íon. O aumento da concentração intracelular de Ca^{2+} ativa a exocitose das vesículas contendo a insulina e o peptídeo C, mediada pelos microtúbulos e proteínas de membrana. Outros substratos metabolizáveis pelas células e que geram ATP, tais como os aminoácidos arginina, lisina e leucina, também estimulam a secreção de insulina (ASHCROFT; RORSMAN, 1989; RUTTER et al., 2015).

A acetilcolina e hormônios como a colecistocinina (CCK) e o peptídeo semelhante ao glucagon 1 (GLP-1) atuam como potencializadores da secreção de insulina (RUTTER et al., 2015). A acetilcolina, por meio do seu receptor M3 (BOSCHERO et al., 1995), e a colecistocinina, pelo receptor GLP1R, ativam a fosfolipase C (PLC), culminando no aumento de inositol trifosfato (IP_3) e de diacilglicerol (DAG). O IP_3 promove a abertura dos canais de Ca^{2+} do retículo endoplasmático ao se ligar ao seu receptor (IP_3R), o que aumenta a concentração citoplasmática desse íon e ativa a maquinaria exocitótica (SHAWL; PARK; KIM, 2009). O DAG ativa a proteína quinase C (PKC), que aumenta a quantidade de vesículas contendo insulina (WAN et al., 2004) e ativa proteínas responsáveis pela translocação dessas vesículas para as proximidades da membrana plasmática (HABER et al., 2001). Já o GLP-1 ativa a adenilato ciclase, que promove a geração de monofosfato cíclico de adenosina (cAMP). O cAMP ativa a proteína quinase A (PKA), que, assim como a PKC, aumenta a quantidade e a mobilidade das

vesículas de insulina, além de permitir a entrada de Ca^{2+} extracelular por meio da fosforilação dos canais de Ca^{2+} sensíveis à voltagem (RORSMAN et al., 2000; TENGHOLM, 2012).

A meia-vida plasmática da insulina é de, aproximadamente, seis minutos; portanto, ela é, na sua maior parte, eliminada da circulação dentro de 10 a 15 minutos. Com exceção da porção da insulina que se liga aos receptores nas células-alvo, o restante é degradado pela enzima que degrada a insulina (IDE), encontrada principalmente no fígado (HULSE; RALAT; WEI-JEN, 2009).

A insulina regula a homeostase de glicose especialmente pela diminuição da sua produção hepática (inibindo enzimas gliconeogênicas e a secreção de glucagon) e aumento da captação desse carboidrato (conduzindo o transportador de glicose, GLUT, até a membrana plasmática), principalmente nos tecidos muscular e adiposo. A insulina também atua nesses tecidos estimulando a síntese proteica e a lipogênese, respectivamente (CARVALHEIRA; ZECCHIN; SAAD, 2002). Para que a insulina consiga desempenhar essas ações, é necessário que ocorra a interação do hormônio com o seu receptor e a ativação de uma série de cascatas de sinalização. A sinalização intracelular da insulina tem início com a sua ligação a um receptor específico de membrana, uma proteína heterotetramérica com atividade quinase, composta por duas subunidades α e duas subunidades β. A ligação da insulina à subunidade α extraceular permite que a subunidade β adquira atividade quinase, levando a alteração conformacional, autofosforilação e consequente ativação (PATTI; KAHN, 1998). Uma vez ativado, o receptor de insulina (IR) fosforila vários substratos proteicos em tirosina, entre eles, os substratos do receptor de insulina (IRS). A partir dos IRS, a sinalização de insulina ocorre por duas principais vias. A primeira envolve a fosfatidilinositol 3-quinase (PI3K), importante na captação de glicose, síntese de glicogênio e de proteínas, bem como na inibição da gliconeogênese e lipólise (FOLLI et al., 1992). A segunda via é a da proteíno-quinase ativada por mitógenos (MAPK), que medeia a expressão gênica e a síntese proteica (PAEZ-ESPINOSA et al., 1999).

Caso ocorra algum problema na síntese, na secreção e na ativação de vias de sinalização de insulina, a glicose não será metabolizada adequadamente, o que pode resultar em hiperglicemia e, caso persista esse quadro, no desenvolvimento do DM.

3.2 Diabetes Mellitus (DM)

O DM foi primeiramente relatado pelos egípcios por volta de 1500 a.C. A doença apresentava como principais aspectos a poliúria e a presença de açúcar na urina, o que foi constatado por se observar que as formigas eram atraídas pela urina dos doentes (MACCRACKEN; HOEL; JOVANOVIS, 1997). Por conta das suas características, a doença recebeu os nomes “diabetes”, palavra de origem grega que significa “sifão”, e “mellitus”, que significa “mel” em latim (LAKHTAKIA, 2010).

A primeira descrição detalhada da doença foi feita pelo grego Aretaeus da Capadócia (150 d.C.). Segundo ele, o DM era uma aflição notável, não muito comum na época. Apesar de levar um período longo até se estabelecer, o enfermo morria rapidamente após a doença se instaurar. A sede era inextinguível e proporcional à quantidade de urina eliminada. Além disso, a emaciação era grande, como se os músculos fossem excretados com a urina (LAIOS et al., 2012).

Em 1798, John Rollo verificou que o açúcar não estava presente apenas na urina dos pacientes diabéticos, mas também no sangue, o que significou se tratar de uma condição sistêmica (MACCRACKEN; HOEL; JOVANOVIS, 1997). Outra descoberta de extrema importância foi feita na década seguinte por Joseph von Mering e Oscar Minkowski. Eles constataram que a remoção do pâncreas de um cachorro levou o animal ao desenvolvimento do DM (MEHRING; MINKOWSKI,1890).

No início do século XIX, Claude Bernard supôs que o glicogênio era armazenado no fígado e que a partir dele era secretado no sangue uma substância “doce”. Foi a superprodução dessa substância, mais tarde chamada de glicose, que o pesquisador considerou como causa do DM (MACCRACKEN; HOEL; JOVANOVIS, 1997).

Atualmente, sabe-se que o DM é uma doença crônica multifatorial, caracterizada por elevadas concentrações sanguíneas de glicose, o que é denominado “hiperglicemia”. Essa hiperglicemia pode ser decorrente da produção insuficiente de insulina ou pela deficiência na sua ação, em que não é possível utilizar efetivamente a insulina produzida (WHO, 2018).

De acordo com a Organização Mundial da Saúde – OMS - (WHO, 2018), cerca de 422 milhões de pessoas em todo o mundo convivem com DM. Além disso, há por volta de 318 milhões de adultos com tolerância à glicose diminuída, o que faz com que esses indivíduos tenham alto risco de desenvolver a doença no futuro (IDF, 2015). Outra informação que justifica a preocupação a respeito do DM é o fato de que em torno de 1,6 milhões de mortes por ano por ano vêm sendo atribuídas às suas complicações (WHO, 2018).

Dentre os tipos de DM, o DM1 e o DM2 são os mais recorrentes. O DM1 representa 5 a 10% dos casos de DM (DANEMAN, 2006; SBD, 2016) e é caracterizado pela deficiência de insulina como consequência da progressiva destruição das células beta pelo sistema imune. A causa exata do DM1 é desconhecida, mas há o consenso de que resulta de uma complexa interação entre os fatores genéticos e ambientais (WHO, 2018). Já o DM2, diagnosticado em 90 a 95% dos casos, pode ser decorrente da resistência à insulina e/ou da deficiência na sua secreção (SALSALI; NATHAN, 2006), e frequentemente está associado ao sedentarismo, à dieta rica em carboidratos e lipídeos e ao envelhecimento. Pode ocorrer em qualquer idade, mas geralmente é diagnosticado após os 40 anos (SBD, 2018).

Para confirmar o diagnóstico do DM, pode-se utilizar, no mínimo, um dos quatro critérios a seguir: glicemia casual ≥ 200 mg/dL, acrescida de poliúria, polidipsia, polifagia e fadiga; glicemia de jejum ≥ 126 mg/dL; glicemia ≥ 200 mg/dL 2 horas após sobrecarga com 75g de glicose e hemoglobina glicada (A1C) ≥ 6,5% (ADA, 2017; SBD, 2018).

O que torna o DM uma doença tão grave são as complicações que podem acometer diferentes locais do organismo, tais como: olhos (cegueira); cavidade oral (periodontite); coração (angina, infarto agudo do miocárdio, insuficiência cardíaca congestiva); cérebro (acidente vascular encefálico); rins (insuficiência renal); nervos (formigamento, perda da sensibilidade) e vasos (ulceração e amputação de membros) (GROSS et al., 2002; WHO, 2018).

Por conta desses agravos, o impacto econômico causado pela enfermidade é gigantesco, uma vez que são necessários recursos para custear tratamentos, internações prolongadas e recorrentes. De acordo com a Federação Internacional do Diabetes (IDF, 2015), o gasto mundial com o DM mais que triplicou no período de 2003 a 2013, e estima-se que atualmente ultrapasse os 827 bilhões de dólares

(WHO, 2016), correspondendo a 12% das despesas globais em saúde (IDF, 2015).

Do ponto de vista social, o diabético pode apresentar redução da capacidade funcional e consequente desempenho prejudicado no trabalho. Aliás, em situações mais graves, como no caso de amputações, o indivíduo pode se tornar dependente de outras pessoas (ADA, 2017). Frente às mudanças que o DM pode significar na vida de quem convive com o problema, é importante levar em consideração o aspecto psicológico e constatar precocemente situações como baixa adesão ao tratamento, desinteresse pelo autocuidado e isolamento social, fatores que aumentam o risco de complicações e pioram o prognóstico da comorbidade (SBD, 2018).

Para evitar o estabelecimento de complicações e amenizar os impactos econômicos, sociais e psicológicos, podem ser adotados tratamentos que abrangem a reeducação alimentar, a prática de atividade física e o uso de medicamentos (IDF, 2015). O transplante de ilhotas pancreáticas também é uma opção para a melhora da qualidade de vida dos pacientes com DM1 que não apresentam controle glicêmico, mesmo fazendo uso de combinações dos métodos supracitados, tanto por proporcionar dieta mais flexível, quanto pela interrupção do incômodo das injeções de múltiplas doses de insulina e das medições diárias de glicemia capilar (FROUD et al., 2005; RYAN et al., 2002; SBD, 2018).

3.2.1 Fisiopatologia do DM1 e DM2

A gênese do DM1 se dá quando infecções virais ou processos inflamatórios promovem a apoptose das células beta e consequente liberação de antígenos. A partir disso, as células apresentadoras de antígeno são ativadas e podem recrutar linfócitos T naive, causadores de infiltração no pâncreas. A infiltração mediada pelas células T leva à geração de EROs e de citocinas pró-inflamatórias como TNF-α, IL-1β e IFN-γ. Esses agentes ativam os fatores de transcrição fator nuclear kappa B (NF-Kb) e transdutor de sinal e ativador de transcrição 1 (STAT-1), que diminuem a expressão do fator promotor de insulina 1 (PDX1) e de GLUT2, reduzindo a produção e secreção de insulina. A ativação de NF-Kb e STAT-1 também desencadeia estresse de retículo endoplasmático, processos apoptóticos e liberação de mais citocinas pelas células beta, levando a um ciclo vicioso de

inflamação e de destruição dessas células que mantém e amplifica o ataque autoimune (HASKINS et al., 2003; VEGA-MONROY; FERNANDEZ-MEJIA, 2012).

A falha na secreção de insulina observada no DM2 pode ser ocasionada pela diminuição da massa e/ou da disfunção das células beta, induzidas pela hiperglicemia prolongada (CANTLEY; ASHCROFT, 2015; POITOUT; ROBERTSON, 2008). Essa condição aumenta a produção de EROs pela cadeia transportadora de elétrons da mitocôndria, os quais ativam vias de morte celular (HUNT; DEAN; WOLFF, 1988; TANAKA et al., 1996). Além disso, a hiperglicemia promove mudanças na expressão dos fatores de transcrição característicos das células beta e diminuição do mRNA, conteúdo e secreção de insulina, o que é denominado desdiferenciação (WEIR; AGUAYO-MAZZUCATO; BONNER-WEIR, 2013). Inclusive, em animais diabéticos já foram observadas células beta que passam a expressar glucagon. Ainda não está claro se essas células são convertidas para alfa ou se há um tipo celular intermediário que expressa tanto glucagon quanto proteínas de células beta (BRERETON et al., 2014).

Outra característica preponderante do DM2, a resistência à insulina, é decorrente da fosforilação de resíduos serina dos IRS, ao invés da fosforilação em tirosina, o que diminui a transdução de sinal a partir desses substratos e consequentemente a ativação das vias PI3K e MAPK (HOTAMISLIGIL et al., 1996; MARTYN; KANEKI; YASUHARA, 2008). Em outras palavras, a insulina não ativa adequadamente suas vias de transdução de sinal, resultando em falha na captação de glicose, na síntese de glicogênio e na inibição da gliconeogênese e lipólise. Em última análise, todos esses eventos culminarão em hiperglicemia (LUCA; OLEFSKY, 2008), e estão relacionados à fosforilação em serina proteínas como a c-jun-N-terminal quinase (JNK), p38 e I-kappa-B-quinase beta (IKKβ). O EO está entre os fatores que levam à ativação dessas quinases (EVANS; MADDUX; GOLDFINE, 2005).

3.3 Estresse oxidativo (EO) e as espécies reativas de oxigênio (EROs)

O estresse oxidativo (EO) é um desequilíbrio entre a produção de espécies moleculares altamente reativas (oxidantes) e as defesas antioxidantes, levando a prejuízos na sinalização redox e/ou a danos moleculares (SIES, 2015). Entre os

agentes oxidantes, merecem destaque as EROs, classificadas em “radicais livres” e “não radicais”. Os radicais livres são átomos, íons ou moléculas que têm oxigênio com um ou mais elétrons não pareados em sua órbita externa, ou seja, o elétron está sozinho no orbital. Exemplos desse grupo são a hidroxila (OH^-), o superóxido (O_2^-) e o hidroperóxido (HO_2^-). No caso dos não radicais, não há elétron não pareado e o principal exemplo é o H_2O_2 (HALLIWELL et al., 1995).

Diferentes compartimentos celulares são responsáveis pela produção das EROs, principalmente a membrana plasmática e a mitocôndria, essa responsável por produzir 90% dessas moléculas (BALABAN; NEMOTO; FINKEL, 2005). O complexo enzimático da NADPH-oxidase presente na membrana plasmática é utilizado pelas células fagocíticas para a eliminação de microrganismos patógenos (BROWNLEE, 2005; NEWSHOLME et al., 2007). Em células como fibroblastos, musculares lisas, endoteliais, mesangiais renais e tubulares renais, esse complexo é a maior fonte de produção de EROs (LI, 2003). Várias subunidades constituem a NADPH-oxidase e em estado inativo algumas ficam localizados no citosol, enquanto outras se situam na membrana plasmática. No citosol são encontradas as subunidades p40fox, p47fox, p67fox e rac, e na membrana plasmática as subunidades p22fox e gp91fox. A fosforilação da subunidade p47fox, principalmente pela PKC, promove a translocação das subunidades citoplasmáticas à membrana e ativa o complexo. A NADPH-oxidase catalisa a doação de um elétron do NADPH para o O_2, formando o O_2^- (Figura 1) (BABIOR, 2004).

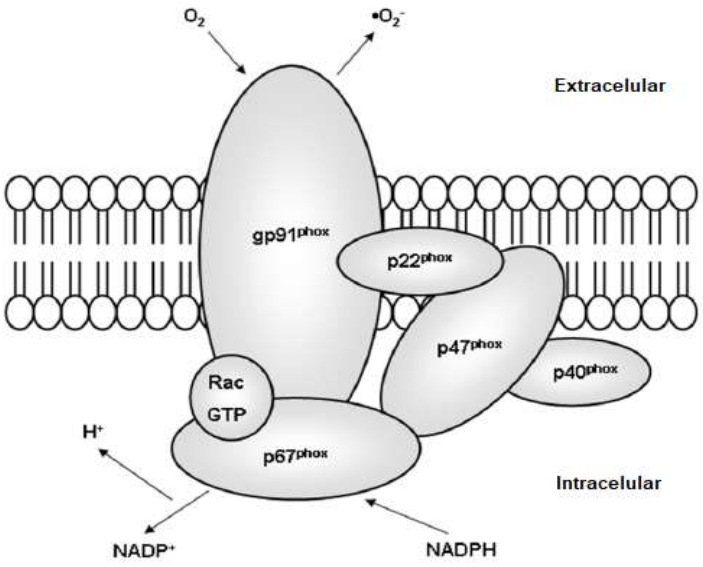


Figura 1. Complexo NADPH-oxidase ativado. Fonte: RABELO L. A. et al. Arquivo Brasileiro de Cardiologia. 2010.

A cadeia transportadora de elétrons está localizada nas cristas da membrana mitocondrial interna, e a formação de EROs a partir dela envolve a atividade dos complexos I, II, III e IV da cadeia respiratória, durante a fosforilação oxidativa. Os elétrons provenientes de vias catabólicas, como do metabolismo de glicose e ácidos graxos pelo ciclo do ácido tricarboxílico, são transferidos para aceptores universais de elétrons: Nicotinamida Adenina Dinucleotídeo (NAD) e a Flavina Adenina Dinucleotídeo (FAD) que são convertidos em NADH e $FADH_2$ quando reduzidos (BROWNLEE, 2005; NEWSHOLME et al., 2007).

O complexo NADH desidrogenase (I) catalisa a transferência dos elétrons do NADH para a coenzima Q. O mesmo processo é realizado pelo complexo succinato desidrogenase (II); porém, nesse caso, quem fornece elétrons é o succinato. O complexo citocromo c-oxidorredutase (III), por sua vez, transporta elétrons da coenzima Q até o citocromo c. Por fim, o complexo citocromo oxidase (IV) conduz elétrons do citocromo c para o oxigênio molecular (O_2), reduzindo-o à água. O fluxo de elétrons é acompanhado pelo bombeamento de prótons através da membrana, produzindo um gradiente eletroquímico ou de voltagem. Esse gradiente impulsiona a síntese de ATP pela ATP sintase. Alternativamente, as proteínas de desacoplamento (UCPs) podem diminuir o gradiente de voltagem para gerar calor, como forma de manter constante a geração de ATP (KORSHUNOV; SKULACHEV; STARKOV, 1997). Uma pequena proporção de elétrons, que não atravessa completamente a cadeia transportadora de elétrons, escapa pelos complexos I e II, reage com o oxigênio e forma o O_2^- (Figura 2).

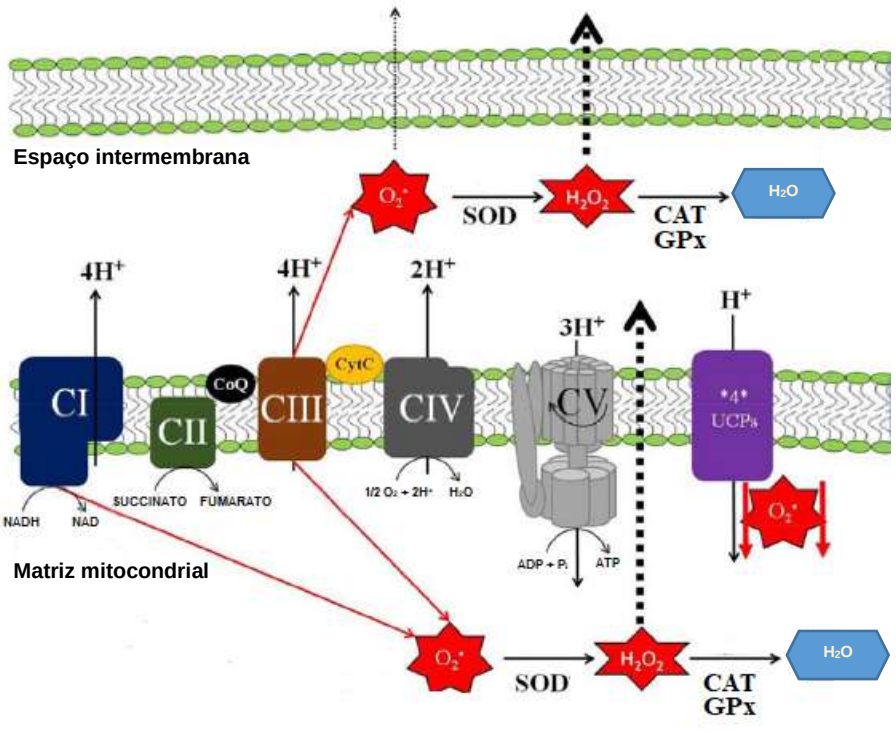


Figura 2. Produção de superóxido pela cadeia transportadora de elétrons mitocondrial. CI: complexo I; CII: complexo II; CIII: complexo III; CIV: complexo IV; CoQ: coenzima Q; CytC: citocromo C; UCPs: proteínas de desacoplamento; SOD: superóxido dismutase; CAT: catalase; GPx: glutationa peroxidase; NADH: Nicotinamida Adenina Dinucleotídeo; O_2^{-}: superóxido; H_2O_2: peróxido de hidrogênio. Adaptado de MC MURRAY F., PATTEN D. A., HARPER, M. E. Obesity. 2016.

A produção de EROs em concentrações fisiológicas, até 100 nM (SIES, 2017), é necessária para a expressão gênica, para a supressão de células tumorais e para a ativação de fatores de transcrição nuclear e de crescimento celular (LEE; KOO; MIN, 2004)**.** As EROs também atuam no mecanismo inflamatório de defesa desempenhado pelas células do sistema imunológico, como os macrófagos e os neutrófilos, visando à eliminação de microrganismos patogênicos. Para isso, as EROs oxidam proteínas, lipídeos e o material gênico do patógeno, induzindo sua morte celular (NATHAN; BUSSEL, 2011; NEWSHOLME et al., 2007).

Para as células beta, as EROs têm importante papel na secreção de insulina. O H_2O_2 exógeno e maleato de dietilo, que aumenta o H_2O_2 intracelular, estimulam a secreção de insulina em linhagem de células beta e ilhotas isoladas de ratos (PI et al., 2007). Acredita-se que esse efeito ocorra pelo aumento do Ca^{2+} intracelular, que estimula a exocitose das vesículas de insulina (JANJIC et al., 1999; MAECHLER; JORNOT; WOLLHEIM, 1999). Por outro lado, a inibição da cadeia transportadora de elétrons por vários agentes, como rotenona, antimicina A,

oligomicina, que aumentam as EROs derivadas de mitocôndrias, mas inibem a geração de ATP, levou à diminuição da secreção de insulina estimulada pela glicose. Ou seja, as EROs participam do mecanismo de secreção de insulina; contudo, por si mesmas não promovem esse efeito. É necessário, principalmente, o aumento da concentração do ATP intracelular pelo metabolismo da glicose (HENQUIN, 2000; WOLLHEIM; MAECHLER, 2002).

Entretanto, quando a concentração de EROs ultrapassa o limite fisiológico em qualquer tipo celular, são observados efeitos deletérios sobre proteínas, lipídeos e DNA. A oxidação de proteínas pode gerar carbonilas, metionina sulfóxido e 2-oxohistidina, compostos que alteram mecanismos de transdução de sinal, sistemas de transporte e atividades enzimáticas (STADTMAN, 2001). Além disso, as proteínas oxidadas são mais suscetíveis à desnaturação e à hidrólise (CECARINI et al., 2007). A bicamada fosfolipídica das membranas celulares é alvo direto da oxidação lipídica, que tem como consequência o aumento da polaridade da fase lipídica e da diminuição da fluidez das membranas e da resistência à desnaturação pela temperatura (LEE; KOO; MIN, 2004; ORRENIUS; GOGVADZE; ZHIVOTOVSKY, 2007). Ademais, a oxidação do DNA pode provocar alterações em suas bases nitrogenadas, como a formação do 8-hidroxiguanosina, utilizado como marcador de dano genético. E a modificação permanente do DNA pode desencadear a mutagênese, carcinogênese e envelhecimento (VALKO et al., 2007).

Algumas condições, como a hiperglicemia, são capazes de acrescer a produção de EROs pelas células beta, principalmente pelo complexo enzimático da NADPH-oxidase e pela mitocôndria (EL-BENNA et al., 2005). O aumento das concentrações intra e extracelulares de glicose induz a formação de EROs pela NADPH-oxidase, pois essa condição aumenta a produção do diacilglicerol (DAG), que estimula a atividade da PKC, e essa é responsável pela ativação do complexo (BROWNLEE, 2001; MORGAN et al., 2007). Com relação à produção de EROs pela mitocôndria, na presença do DM, há mais glicose e lipídeos sendo oxidados no ciclo do ácido tricarboxílico, o que leva mais NADH e succinato para a cadeia respiratória dessa organela. Como resultado, o gradiente de voltagem da membrana mitocondrial aumenta até atingir um limiar crítico. A partir daí a transferência de elétrons no interior do complexo III é bloqueada, fazendo com que eles retornem à coenzima Q, a qual doa os elétrons para o O_2, aumentando a

produção de O_2^-.

3.3.1. Modelos de indução de estresse oxidativo

Para melhor compreensão da gênese do EO e como ele se relaciona com as mais diversas doenças, podem ser utilizados compostos, tanto *in vivo* quanto *in vitro*, que induzem a formação das EROs e/ou mimetizam sua ação, como: dieta *high-fat* (60% lipídeos, 20% proteínas e 20% carboidratos) (TACHIBANA et al., 2015); elevada concentração de carboidratos como ribose (BENSELLAM et al., 2015) e glicose (LIU et al., 2014); ácido palmítico (SHEN et al., 2014), terc-butil hidroperóxido (t-BHT) (FERNÁNDEZ-MILLÁN et al., 2014) e peróxido de hidrogênio (H_2O_2) (FIORY et al., 2014; LEE et al., 2009), sendo que esse último foi utilizado neste trabalho.

O H_2O_2 é uma ERO não radical extremamente abundante em organismos aeróbicos e relativamente estável *in vivo,* se comparado a outras EROs. Sua meia vida em linfócitos, por exemplo, é 1 ms, enquanto do O_2^- é 1 µs (RETH, 2002). O H_2O_2 entra nas células por meio das aquaporinas e é capaz de se difundir pelas membranas celulares (BIENERT; SCHJOERRING; JAHN, 2006). Sua concentração fisiológica é entorno de 10 nM (SIES, 2014) e o acréscimo da sua produção faz com que oxide proteínas, principalmente nos resíduos cisteína (BIENERT; SCHJOERRING; JAHN, 2006; RETH, 2002). O H_2O_2 induz danos no DNA via formação de OH^-, radical altamente reativo, pela sua reação com metais de transição (SLAMENOVA et al., 2013).

3.3.2 Defesas antioxidantes

Para que possa ocorrer equilíbrio entre a produção e a eliminação das EROs, é necessário que as células tenham sistemas de defesa antioxidante que abrangem enzimas como a CAT, a glutationa peroxidase (GPx) e o superóxido dismutase (SOD) (VALKO et al., 2007). Existem oito isoformas conhecidas de GPx, sendo a GPx1 (citosólica) a mais abundante (MEHMETI et al., 2017; PAPP et al., 2007) e três isoformas de SOD, a SOD1 (citosólica, CuZn SOD), a SOD2 (mitocondrial, Mn

SOD) e a EC-SOD (extracelular) (MARITIM; SANDERS; WATKINS, 2003; MARKLUND, 1982). O O_2^- proveniente, principalmente, de organelas como a membrana plasmática e a mitocôndria é dismutado pela SOD, formando o H_2O_2. O H_2O_2 pode ser metabolizado pela CAT ou pela GPx. No primeiro caso, ocorrerá a produção de uma molécula de H_2O e O_2 e no segundo, duas moléculas de H_2O (LEE; KOO; MIN, 2004). O H_2O_2 pode ainda reagir com metais de transição como Fe^{2+} ou Cu^{2+} (reação de Fenton) e com o superóxido (Reação de Haber-Weiss), formando a mais deletéria das EROs, a OH^- (HALLIWELL; GUTTERIDGET, 1984). Para esse radical livre; entretanto, não há enzimas que promovem a sua metabolização, por isso, provoca-se extensa destruição tecidual (LEE; KOO; MIN, 2004)**.**

Em comparação com outros tecidos, as células beta pancreáticas apresentam menor expressão da CAT, GPx e SOD. Por exemplo, a expressão de SOD nas células beta e da CAT e GPx nas ilhotas corresponde a 50% e 1%, respectivamente, da observada no tecido hepático (LENZEN; DRINKGERN; TIEDGE, 1996; TIEDGE et al., 1997). Isso poderia contribuir para a suscetibilidade das células secretoras de insulina à danos mediados pelo EO (LENZEN; DRINKGERN; TIEDGE, 1996).

Normalmente, as enzimas antioxidantes não estão expressas na sua capacidade máxima, mas são altamente induzíveis pelos fatores de transcrição. Fatores de transcrição são proteínas que podem ativar ou reprimir a transcrição de um gene por meio de sua interação com sequências específicas do DNA (VILLARD, 2004). A ativação clássica da transcrição das enzimas antioxidantes envolve a via de sinalização Keap1-Nrf2-ARE. Keap1 (*Kelch-like ECH-associated protein 1*) é uma proteína repressora que retém o Nrf2 (fator nuclear eritroide 2 relacionado ao fator 2) no citoplasma e estimula a sua degradação proteassomal. Substâncias oxidantes e eletrófilos, por exemplo, podem reagir com resíduos de cisteína da Keap1, provocando a sua alteração conformacional e a liberação do Nrf2. O Nrf2 migra para o núcleo onde forma um heterodímero com proteínas da família *small* Maf, o qual se liga ao ARE (elementos de resposta antioxidante). A transativação de ARE permite recrutar elementos transcricionais para a expressão dos genes regulados (ITOH et al., 1999; KENSLER; WAKABAYASHI; BISWAL, 2007; SYKIOTIS; BOHMANN, 2010).

Apesar de esse mecanismo ser amplamente estabelecido, outras vias de

sinalização parecem ter influência sobre a transcrição das enzimas antioxidantes, como a via dos receptores ativados por proliferadores de peroxissoma (PPARs). Os PPARs são fatores de transcrição da família dos receptores nucleares que regulam diversos eventos biológicos com destaque para diferenciação e desenvolvimento celular, homeostase da glicose, metabolismo de lipídeos e inflamação. Estão presentes em três subtipos: α, β/δ e γ (BERGER; MOLLER, 2002). O PPAR α é expresso predominantemente no fígado, e em menor abundância no coração e no rim; o PPAR β/δ é ubíquo, embora a sua expressão seja maior no tecido adiposo, na pele e no músculo esquelético (SEMPLE; CHATTERJEE; RAHILLY, 2006); o PPAR γ tem três isoformas. O PPARγ1 é expresso em ampla variedade de tecidos incluindo coração, músculo, rim, baço e pâncreas; o PPARγ2 está presente no tecido adiposo; e o PPARγ3, em macrófagos e no intestino grosso (TYAGI et al., 2011).

Os PPARs ativam a transcrição de genes a partir da formação de um heterodímero com o receptor x retinoide (RXR), que se liga a sequências específicas do DNA, chamadas de elementos responsivos aos proliferadores de peroxissoma (PPRE), na região promotora dos genes alvo. Na ausência de um ligante, o complexo PPAR/RXR se mantém ligado a co-repressores transcricionais que impedem sua interação com o PPRE. Na presença de um ligante agonista, uma alteração conformacional leva à liberação do correpressor, recrutamento de coativadores, transativação de promotores relacionados ao PPRE e indução da transcrição (DEBRIL et al., 2001). Os coativadores e os correpressores transcricionais existem em complexos multiproteicos contendo enzimas modificadoras de histonas, tais como histonas acetiltransferases e histonas deacetilases. A atividade dessas enzimas modificadoras de histonas afeta a transcrição gênica por alterar a estrutura da cromatina (MCKENNA; MALLEY, 2002).

Atuam como ligantes do complexo PPAR/RXR tanto substâncias endógenas como esteroides, ácidos graxos, vitaminas e hormônios (REPA; EVANS; MANGELSDORFZI, 2001), quanto sintéticas, como os fibratos e as tiazolidinedionas (TZDs) (BERGER; AKIYAMA; MEINKE, 2005).

Os fibratos, ligantes do PPARα como o fenofibrato, são prescritos principalmente para o tratamento da hipertrigliceridemia (SAHEBKAR et al., 2017) e na prevenção da aterosclerose (SAHEBKAR et al., 2016). Esse fármaco também

atua sobre as células beta pancreáticas, restabelecendo a secreção de insulina prejudicada pelas EROs (SUN et al., 2008) e diminuindo a hiperglicemia de animais com diabetes induzido pelo estresse oxidativo, por aumentar a atividade da CAT e SOD (YARIBEYGI et al., 2018).

As TZDs, ligantes de PPARγ, como a pioglitazona, são indicadas para o tratamento do DM2 em humanos por aumentarem a captação de glicose estimulada pela insulina no músculo esquelético e tecido adiposo, por inibirem a produção hepática de glicose e por reduzirem a hipertrigliceridemia e a concentração de ácidos graxos livres no plasma (DEBRIL et al., 2001; SEMPLE; CHATTERJEE; RAHILLY, 2006). Em modelos animais diabéticos db / db e obesos pré-diabéticos, as TZDs aumentam a proliferação e reduzem a apoptose de células beta (KANDA et al., 2010; ZENDER et al., 2004) e estimulam a secreção de insulina (HIGA et al., 1999), respectivamente. Diante do estresse oxidativo, a pioglitazona previne a formação de EROs, protegendo as ilhotas pancreáticas e as células beta da apoptose (SAITOH et al., 2008; VANDEWALLE et al., 2008). Nesse sentido, os promotores dos genes das enzimas antioxidantes têm PPRE (CHUNG et al., 2011; GIRNUN et al., 2002; YOO; CHANG; RHO, 1999) e estudos mostram que as TZDs aumentam a expressão de CAT, GPx e SOD2, tanto em ilhotas quanto em células beta (KANDA et al., 2010; CHUNG et al., 2011).

3.4 Prolactina

Descoberta em 1960, a Prolactina (PRL) é um hormônio constituído por 199 aminoácidos e tem aproximadamente 23 kDa. É secretada, predominantemente, pela hipófise anterior (FRIESEN; GUYDA; HARDY, 1970; GUELHO et al., 2016), apesar de também ser verificada a sua produção em linfócitos, glândula mamária, glândula prostática, fibroblastos cutâneos e tecido adiposo (GOUVEIA et al., 2014).

Normalmente, a liberação da PRL é controlada pelo hipotálamo, que exerce sobre ela uma inibição por meio da secreção de dopamina na eminência média. Por esse motivo, as drogas que interferem na síntese ou na ação da dopamina aumentam a secreção de PRL (LYONS; BROBERGER, 2014).

A secreção da PRL pode ser estimulada por situações de estresse, como cirurgia, medo, exercício físico e sono, embora elevada concentração desse

hormônio seja encontrada principalmente durante o período gestacional. Homens e mulheres apresentam concentrações séricas basais normais semelhantes (FREEMAN et al., 2000).

Uma vez na corrente sanguínea, a PRL não se liga a proteínas séricas e, por essa razão, tem uma meia-vida relativamente curta de cerca de 20 minutos. A transdução de sinal a partir da PRL ocorre após a ligação do hormônio ao seu receptor (Prlr), causando a sua dimerizacão e consequente ativação da janus quinase 2 (JAK2). A JAK2, por sua vez, fosforila os resíduos de tirosina do domínio intracelular do receptor, proporcionando locais de ligação para diversas proteínas sinalizadoras, destacando-se as transdutoras de sinal e ativadoras de transcrição (STAT), particularmente as STAT5. As STATs, como fatores de transcrição citoplasmática latentes, se separam do complexo e migram até o núcleo, onde ativam a transcrição gênica de proteínas (IGNACAK et al., 2012).

Apesar de a PRL ter sido associada inicialmente à estimulação da produção de leite, atualmente apresenta dezenas de ações reconhecidas, entre elas: diminuição da excreção renal de sódio e potássio (IBARRA et al., 2005); aumento da absorção intestinal de água e sais; influência na resposta ao estresse e à ansiedade; regeneração do sistema nervoso central (MÖDERSCHEIM et al., 2007); lipogênese (STEWART et al., 2004); proteção contra o desenvolvimento de cardiomiopatia pós-parto (HILFIKER-KLEINER et al., 2007); estimulação ou inibição da resposta imune de acordo com sua concentração (TOMIO et al., 2008), além de diversos efeitos sobre o pâncreas.

Durante o período de gestação, ocorrem alterações morfofisiológicas nas células beta de humanas e ratas, dentre elas o aumento da massa celular e da secreção de insulina (BRELJE et al., 1993; SORENSON; BRELJE, 1997) como mecanismo adaptativo para compensar a resistência à insulina que é verificada nessa fase. O aumento da massa da ilhota pancreática pode alcançar 50% e se deve pela hipertrofia e hiperplasia de células e redução da morte celular (AMARAL et al., 2004).

O Prlr está presente nas ilhotas pancreáticas e a sua expressão aumenta durante a prenhez/gravidez. Em estudo realizado com camundongos que não apresentavam o Prlr, verificou-se hipoplasia das ilhotas e das células beta e o surgimento de intolerância discreta à glicose (FREEMARK et al., 2002). Amaral e colaboradores (2004) demonstraram que proteínas das vias PI3K e MAPK têm

expressão aumentada em ilhotas de ratas prenhas e grande parte desse efeito é dependente do Prlr. Esses achados confirmam que os ligantes desse receptor, dentre eles a PRL, estão associados ao aumento da massa e da sensibilidade à glicose das ilhotas de ratas, observados durante a prenhez.

Um efeito muito semelhante é observado em ilhotas de ratos neonatais tratadas com PRL, em que ocorre aumento da secreção de insulina estimulada pela glicose (BOSCHERO et al., 1992), da fosforilação dos IRS 1 e 2 (AMARAL et al., 2003), da expressão proteica do GLUT2 (DE MAZANCOURT et al., 1994) e de proteínas das vias PI3K e MAPK (AMARAL et al., 2003).

Diante desses resultados, somados ao fato de a PRL melhorar a viabilidade de células beta pancreáticas humanas em cultura (YAMAMOTO et al., 2009), passou-se a investigar se a PRL poderia ser uma alternativa para aumentar a eficácia do transplante de ilhotas. Apesar de o transplante de ilhotas ter emergido como um tratamento alternativo para o DM1 (RYAN et al., 2002), a perda de células saudáveis durante o processo de isolamento das ilhotas é um fator que limita o sucesso da técnica. Como elas são avasculares e, portanto, isquêmicas desde o isolamento até ao período necessário para a revascularização, algumas substâncias nocivas podem ser produzidas, como as EROs e citocinas pró-inflamatórias, causando prejuízos no funcionamento das células e/ou induzindo a apoptose (MONFARED; LARIJANI; ABDOLLAHI, 2009).

O tratamento de ilhotas de camundongos com PRL, precedendo o transplante, fez com que animais diabéticos se tornassem normoglicêmicos mais rapidamente, bem como tivessem aumento da vascularização pela redução da expressão do inibidor da angiogênese, TSP-1 (JOHANSSON et al., 2009). Além disso, a pré-incubação de ilhotas de humanos com PRL aumentou a proliferação e diminuiu a apoptose de células beta induzida por citocinas pró-inflamatórias e H_2O_2, após o transplante (YAMAMOTO et al., 2010). A PRL protege as células beta contra a apoptose induzida por citocinas por aumentar a expressão de proteínas anti-apoptóticas da família BCL2 (TERRA et al., 2011) e prevenir a ativação da via JNK e da NF-Kb (NARDELLI et al., 2018). Entretanto, ainda é desconhecido o mecanismo pelo qual esse hormônio protege as células beta do estresse oxidativo.

Nesse sentido, é possível que a PRL possa modular a expressão de enzimas antioxidantes, visto que o aumento da expressão gênica da SOD1 e da EC-SOD foi verificado em ilhotas de ratas e em linhagem celular de fibroblastos,

respectivamente, tratados com esse hormônio (ADACHI et al., 2004; BORDIN et al., 2004). Além do mais, a PRL exerce efeito protetor sobre células da retina submetidas a estresse oxidativo, embora não esteja completamente esclarecido de que maneira isso ocorre. Em camundongos sem a atividade do Prlr ocorreu a diminuição do mRNA da CAT nas células da retina. E quando realizado tratamento com PRL em camundongos com receptor ativo, o hormônio aumentou a atividade da GPx (THÉBAULT, 2017). Ademais, em linhagem celular de pré-adipócitos e em tecido adiposo de ratos, a PRL aumentou a expressão gênica do PPARγ (NANBU-WAKAO et al., 2000), que tem influência sobre a expressão de enzimas antioxidantes.

4. METODOLOGIA

4.1 Cultivo celular e tratamento

Células beta pancreáticas da linhagem INS-1E, gentilmente cedidas pelo Prof. C. Wolheim (Centro Médico Universitário, Genebra, Suíça), foram cultivadas em meio RPMI 1640 (Vitrocell®, SP, Brasil) e suplementadas com 5% v/v de soro fetal bovino (FBS), HEPES 10 mmol/L, piruvato de sódio 1 mmol/L e 2-mercaptoetanol 50 µmol/L com 11 mmol/L de glicose. As células foram semeadas em placas de 96 poços (15.000 ou 50.000 células por poço), em triplicata, para avaliação da viabilidade celular, mitoSOX e mito-roGFP2-Orp1 e em placas de 24 poços (100.000 células por poço) para os demais experimentos. Para o tratamento com PRL e H_2O_2, as células foram mantidas em meio sem FBS, com 1% p/v de BSA e 5,6 mmol/L de glicose. Os experimentos foram realizados 24h após o pré-tratamento com PRL (0,5 µg/mL; Programa Nacional de Hormônio e Peptídeos, #AFP7547B) ou veículo ($NaHCO_3$ 0,01 mmol/L + BSA 0,3% p/v) e subsequente exposição a H_2O_2 (Sigma, H1009). A PRL e o veículo foram mantidos no meio de tratamento durante a exposição ao H_2O_2. Células INS-1E expostas apenas à PRL foram usadas no experimento de time-course.

4.2 Avaliação da viabilidade celular

A viabilidade celular foi avaliada a partir de: a) células coradas com intercalantes de DNA Hoechst 33342 (ThermoFisher, H3570; 10 µg/mL) e iodeto de propídio (ThermoFisher, P1304MP; 5 µg/mL). Foram removidos 100 µL do meio de tratamento de cada poço e adicionados 100 µL de meio de cultura contendo os corantes Hoechst 33342 (ThermoFisher, H3570; 10 µg/mL) e iodeto de propídio (ThermoFisher, P1304MP; 5 µg/mL). Após 15 min de incubação, a 37 ºC, foram removidos 100 µL do meio de cada poço e adicionados 100 µL de meio RPMI-1640. A porcentagem de células necróticas/apoptóticas foi determinada em High Content Imaging System (ImageXpress; Molecular Devices) por meio do módulo *Live and Dead* do software MetaXpress (Molecular Devices). Para identificar as populações de células viáveis e mortas, foram aplicadas máscaras para os comprimentos de onda DAPI (excitação em 350 nm e emissão em 470 nm; Hoescht) e Texas *red* (excitação em 596 nm e emissão em 615 nm; iodeto de propídio); b) Western blot da caspase 3 clivada (ver detalhes abaixo).

4.3 Western Blot

As células foram lavadas com PBS 1x e coletadas com 60 µL de tampão de extração de proteínas Laemmli. As amostras foram aquecidas a 100 °C, por 5 min, para posterior aplicação de 20 µL de seu conteúdo em SDS-PAGE com 12% de acrilamida. Após separação eletroforética em aparatos, as proteínas foram transferidas por transferência líquida, com 20% de metanol, para membrana de nitrocelulose (com poro de 0,22 µm), e o bloqueio de ligações inespecíficas foi realizado com tampão TBS-T (50 mM Tris pH 7,5, NaCl 150 mM, 0,1% Tween 20) contendo 10% de leite desnatado, durante 60 min. As membranas foram incubadas durante 16h, a 4 °C, com anticorpos primários para a α-tubulina (Santa Cruz, 8095), β-actina (Santa Cruz, 81178), caspase 3 clivada (Abcam, 4051), catalase (Abcam, 16731), glutationa peroxidase 1 (abcam, 22604), superóxido dismutase 1 (abcam, 16831) e superóxido dismutase 2 (Santa Cruz, 30080). Em seguida, as membranas foram incubadas por 60 min com anticorpos secundários conjugados com peroxidase, em temperatura ambiente. O conteúdo proteico foi detectado pelo

ImageQuant LAS 4000 (Healthcare Bio-Sciences) após a reação de quimiluminescência com o Substrato Quimioluminescente SuperSignal West Femto (Thermo Scientific). Finalmente, a densitometria das bandas de proteínas foi determinada com o software ImageQuantTL (Healthcare Bio-Sciences). Os valores obtidos foram normalizados pelos controles internos, α-tubulina ou β-actina.

4.4 PCR quantitativa em tempo real (qRT-PCR)

A extração do RNA total foi realizada com o reagente TRIzol (Invitrogen, 15596026), de acordo com as instruções do fabricante. Em seguida, o RNA das amostras foi quantificado no leitor multimodal Spectramax i3 (Molecular Devices). A síntese de cDNA foi feita por meio do kit *High Capacity cDNA Reverse Transcription* (Applied Biosystems, 4668814) e a amplificação de cDNA foi realizada com o reagente *Power SYBR Green Master Mix* (Applied Biosystems, 4367659), juntamente com os primers para *pparg* (Senso, 5´-CCTTGCTGTGGGGATGTCTC-3´; anti-senso, 3´-CGCCAACAGCTTCTCCTTCT-5´) e para o gene controle, rps 26 (Senso, 5´-TTTTTCCTCCTTGGGCGTCTG-3´; anti-senso, 3´-GGTAGACAGTCGAATCATCCATTCA-5´). O qRT-PCR foi realizado com o *Applied Biosystems StepOne real-time PCR system* (Applied Biosystems) e a expressão do mRNA foi determinada após a normalização pelo método 2-ΔΔCT (LIVAK; SCHMITTEGEN, 2001).

4.5 MitoSOX

As células foram incubadas durante 20 min com a sonda mitoSOX (Invitrogen, M36008; 5 µg*/*mL) e o corante intercalante de DNA Hoechst 33342 (ThermoFisher, H3570; 5 µg*/*mL). O mitoSOX é um indicador de O_2^- mitocondrial, uma vez que sofre oxidação por essa espécie reativa e é convertido em um produto fluorescente. A aquisição da fluorescência foi realizada de acordo com as instruções do fabricante, em um leitor de microplacas, utilizando o *High Content Imaging System* (ImageXpress; Molecular Devices), com o módulo de análise *Cell Scoring of MetaXpress* (Molecular Devices). Apenas a fluorescência do mitoSOX

no citoplasma foi considerada, uma vez que essa sonda migra para o núcleo das células em processo de apoptose (LIVAK; SCHMITTEGEN, 2001). Os valores obtidos foram normalizados pelo número de células, determinado pela fluorescência do corante Hoechst.

4.6 Mito-roGFP2-Orp1

Células INS-1E expressando estavelmente a sonda mito-roGFP2-Orp1 foram obtidas por transdução retroviral. Resumidamente, células Phoenix-AMPHO foram transfectadas com pLPCX/mito-roGFP2-Orp1 (codificando a sonda roGFP2-Orp1 com uma sequência de direcionamento mitocondrial N-terminal) pelo método do fosfato de cálcio. Após 6h de transfecção, as células foram lavadas duas vezes com PBS e transferidas para meio fresco. Após 24 e 48h de transfecção, o sobrenadante foi recolhido e passado através de um filtro de 0,22 mm. As células INS-1E (semeadas a 2,7 x 10^5 células por poço em uma placa de seis poços, durante 24h antes de cada experimento) foram incubadas com o sobrenadante contendo o vírus, suplementado com Polybrene, (4 mg/mL) a 37°C por 24h , em uma atmosfera de 5% de CO_2. As células foram então cultivadas por vários dias em meio contendo puromicina (0,5 mg/mL). Uma população celular altamente fluorescente (~ 6% da população total) foi selecionada por citometria de fluxo (FACSAria). As células coletadas foram expandidas no meio contendo puromicina (0,5 mg/mL) e gentamicina (50 mg/mL). Imagens com um microscópio de fluorescência foram capturadas para confirmar a localização mitocondrial da sonda. A mito-roGFP2-Orp1 é uma sonda sensível ao H_2O_2 com dois picos de excitação diferentes e redox dependes, em 405 nm e 488 nm, e um único pico de emissão, em 510 nm. Enquanto a oxidação da sonda por H_2O_2 resulta em um aumento do pico de excitação de 405 nm, a redução da sonda aumenta a excitação em 488 nm. Consequentemente, a razão entre 405/488 nm pode ser usada como um indicador da quantidade relativa de mito-roGFP2-Orp1 oxidada/reduzida e da concentração mitocondrial de H_2O_2 (MORGAN, SOBOTTA, DICK, 2011). Após o pré-tratamento, as células foram expostas ao H_2O_2 e imediatamente submetidas à leitura de fluorescência em 405 e 480 nm, por cerca de 1h, pelo CLARIOstar (BMG LABTECH). Foram analisadas fluorescências obtidas 10 min após a adição de

H_2O_2, em um intervalo de 10 min. A razão entre as fluorescências (400/480) foi normalizada pela razão entre fluorescências da sonda maximamente reduzida e maximamente oxidada, mensurada após a adição de diidrotreitol (10 mM) e diamida (2 mM).

4.7 Ensaio *in silico*

Foram obtidos dados de transcriptoma a partir do macroarray de 1176 genes, previamente realizado por nosso grupo de pesquisa, utilizando ilhotas de ratas Wistar (90 a 120 dias de vida; controle) e ilhotas de ratas Wistar prenhes (15º dia de gestação). As análises subsequentes foram realizadas com os genes que apresentaram diferenças relativas acima de 2 *fold change* em relação ao grupo controle e com $p < 0,05$. Esses dados foram importados para o software DAVID (*Database for Annotation, Visualisation and Integrated Discovery v6.8*), no qual foi avaliado o enriquecimento das vias KEGG (*Kyoto Encyclopedia of Genes e Genomes*). Também analisou-se o enriquecimento de ontologia de genes por "componentes celulares" através do GOterms-CC (http://www.geneontology.org).

4.8 Ensaio de promotor repórter

As células foram transfectadas por 16h utilizando meio RPMI 1640, meio Opti-MEM (Gibco, 31985070) e Lipofectamina 3000 (Invitrogen, L3000015) com 0,5 mg de pRL-SV40 (luciferase renilla; Promega, E2231) e 30 ng de pPPRE X3- TK-luciferase (elemento responsivo ao PPAR - PPRE; Dr. Bruce Spiegelman, Addgene, 1015) por poço. Em seguida, o meio de transfecção foi substituído pelo meio RPMI 1640 e após 24h as células foram tratadas com veículo ou PRL (0,2 µg/mL, 0,5 µg/mL ou 1,0 µg/mL) durante esse mesmo período. As amostras foram coletadas com 100 µL de tampão de lise (Promega, E1500) e a atividade da luciferase foi mensurada em 20 µL do lisado em placa branca de 96 poços (costar), utilizando o sistema Dual luciferase assay (Promega) de acordo com as instruções do fabricante. Por fim, os valores obtidos da atividade de pPPRE X3-TK-luciferase foram normalizados pela atividade da renilla luciferase.

4.9 RNA de interferência (siRNA)

Dois siRNAs diferentes para o PPARγ (siPPARγ-1 e siPPARγ-2; Sigma-Aldrich) foram usados para reduzir a expressão desse gene nas células INS-1E. *AllStars Negative Control* siRNA (Qiagen) foi utilizado como controle negativo. As células foram incubadas por 16h com meio RPMI 1640, meio Opti-MEM e lipofectamina RNAiMAX (Invitrogen, 13778075) com 40 nM de siPPARγ e 40 nM do siRNA *AllStars Negative Control* (Qiagen, 1027281). Em seguida, o meio de transfecção foi substituído pelo meio RPMI 1640 e após 24h foi realizado o tratamento.

4.10 Análise estatística

Os resultados foram expressos como a média ± erro padrão da média (EPM). Para a avaliação estatística, utilizou-se o teste t de Student e ANOVA (*one or two way*), seguido de pós-teste de Bonferroni. O nível de significância adotado foi de $p < 0,05$ e as análises foram feitas por meio do software GraphPad Prism 6.00. O número de amostras utilizadas foi descrito na legenda dos gráficos. No método *in silico*, os valores de corte usados para a análise de enriquecimento de vias KEGG foi de $p < 0,1$ e *Fold enrichment* $p \geq 1,5$ (HUANG; SHERMAN; LEMPICKI, 2009). Os resultados dessa análise foram apresentados em *-log Fold Change.*

5. RESULTADOS E DISCUSSÃO

ARTIGO: Prolactina aumenta a expressão de enzimas antioxidantes e ativa o receptor ativado por proliferador de peroxissoma (PPAR) em células INS-1E

Prolactin increases the expression of antioxidant enzymes and activates peroxisome proliferator-activated receptor (PPAR) in INS-1E cells

Carine Marmentini[1], Daniel Andrade da Cunha[2], Tarlliza Romana Nardelli[3], Tanes Imamura de Lima[3], Leticia Prates Roma[4] Silvana Bordin[5], Sandra Lucinei Balbo[1], Antonio Carlos Boschero[3], Emerielle Cristine Vanzela[3*]

[1]Laboratory of Endocrine Physiology and Metabolism, Center for Biological and Health Sciences, State University of Paraná West, Cascavel, Brazil

[2]Center for Diabetes Research, Faculty of Medicine, Université Libre de Bruxelles, Brussels, Belgium

[3]Laboratory of Endocrine Pancreas and Metabolism, Institute of Biology, University of Campinas, Campinas, Brazil

[4]Biophysics Department, Center for Human and Molecular Biology, Universität des Saarlandes, Homburg/Saar, Germany

[5] Department of Physiology and Biophysics, Institute of Biomedical Sciences, University of Sao Paulo, Sao Paulo, Brazil

***Correspondence:**
Emerielle Cristine Vanzela, PhD
Laboratory of Endocrine Pancreas and Metabolism, Institute of Biology, University of Campinas, Campinas, Brazil, ZIP: 13083-864
Phone: + 55 19 3521 0010
ecv@unicamp.br

Abstract

Oxidative stress causes functional damage to pancreatic beta cells and contribute to the development of diabetes mellitus. Prolactin, a hormone highly secreted in the gestational and lactation period, has known beneficial effects on insulin-secreting cells, including the protection of beta cells against H_2O_2-induced cell death, although the mechanism is still unknown. In this sense, prolactin modulates the expression of antioxidant enzymes in pancreatic islets, retinal cells and fibroblasts. Thus, we investigated whether the protection of prolactin in pancreatic beta cells against oxidative stress occurs by an increase of antioxidant enzymes and which pathway would be involved in this effect. We found that prolactin increases the protein content of antioxidant enzymes and decreases mitochondrial concentration of H_2O_2 in INS-1E cells. Bioinformatics analyzes of genes upregulated in islets of pregnant rats revealed enrichment of the peroxisome proliferator-activated receptor (PPAR) pathway, which is involved in the expression of antioxidant enzymes in different cell types. Performing a promoter reporter assay we found that PRL activates PPAR. Next, we evaluated whether PRL would be dependent on PPARγ to avoid cell death caused by H_2O_2, once PPARγ expression is increased by PRL in pre-adipocyte cell line and rat adipose tissue. However, the knockdown of this transcription factor did not alter the efficiency of PRL in protecting beta cells from oxidative stress. For the first time we demonstrated that PRL increases the production of antioxidant enzymes in INS-1E cells and activates a transcription factor important for the functioning of pancreatic beta cells.

Keywords

Pancreatic beta cell; Oxidative stress; Cell death; Prolactin; PPAR; Diabetes

Highlights

PRL exerts a protective effect against H_2O_2-induced beta cell death

Antioxidants enzymes protein content are increased by PRL

PRL decreases mitochondrial concentration of H_2O_2

PRL actives PPAR

1. Introduction

Diabetes mellitus (DM) is a chronic disease characterized by permanent hyperglycemia [1] caused by defects in insulin secretion [2], insulin action, or both [3]. Oxidative stress, an increase in the ratio between highly reactive molecular species and antioxidant defenses [4], is among the factors that cause functional damage to pancreatic beta cells and contribute to the development of the disease [5-8].

Among the reactive molecular species, the oxygen species (ROS) are the most abundant in the cells and have as representatives the hydroxyl (OH^-), superoxide (O_2^-), hydroperoxide (HO_2^-) and hydrogen peroxide (H_2O_2) [4]. To maintain the physiological concentration of ROS and avoid oxidative stress, the cells have antioxidant defense systems that include enzymes such as catalase (CAT), glutathione peroxidase (GPx) and superoxide dismutase (SOD) [9].

Prolactin (PRL), is a protein hormone secreted predominantly by adenohypophysis. High concentration of this hormone is observed during the gestational period [10-12]. In this phase, morphophysiological alterations in the pancreatic beta cells of human and rats are verified, among them the increase of the cellular mass and the insulin secretion [13,14] as an adaptive mechanism to compensate the insulin resistance that occurs in pregnancy and the PRL receptor is related to these alterations [15]. In cell culture, in addition to the previously mentioned effects [16,17], PRL has also been shown to protect against cell death induced by proinflammatory cytokines and H_2O_2 [18]. Recently, pathways involved in the protection of beta cells by PRL against insult caused by pro-inflammatory cytokines have been described [19]. However, the mechanism by which this hormone protects beta cells from oxidative stress remains unknown. In this sense, studies have already shown that PRL increases the gene expression of SOD in pancreatic islets [20] and in fibroblast cell line [21]. In retinal cells of mice subjected to oxidative stress, the absence of the PRL receptor decreased the mRNA of CAT [22].

In this way, we investigate whether the protection exerted by prolactin against oxidative stress is due to the increased production of the antioxidant enzymes and whhicht pathway would be involved in this effect. Our results demonstrated for the first time that PRL increases the protein content of antioxidant enzymes, prevents the mitochondrial concentration of H_2O_2 and activates the transcriptional factor peroxisome proliferator-activated receptor (PPAR) in INS-1E beta cell line.

2. Material and Methods

2.1 Cell culture and treatment. Insulin-producing INS-1E cells, kindly given by the Prof. C. Wolheim (Centre Médical Universitaire, Geneva, Switzerland), were cultured in RPMI 1640 medium (Vitrocell®, SP, Brazil) and supplemented with 5% v/v of fetal bovine serum (FBS), HEPES 10 mmol/L, sodium pyruvate 1 mmol/L and 2-mercaptoethanol 50 μmol/L with 11 mmol/L glucose. The cells were seeded in 96-well plates (15,000 cells by well or 50,000 cells), in triplicate, to the assessment of cell viability, mitoSOX and mito-roGFP2-Orp1 and in 24-well plates (100,000 cells by well) to the others experiments. For the PRL and H_2O_2 treatment, the cells were maintained in a medium with 1% w/v of BSA and 5.6 mmol/L glucose without FBS. The experiments were performed 24h after pretreatment with PRL (0.5 μg/mL; National Hormone and Peptide Program, #AFP7547B) or vehicle (0.01 mmol/L NaHCO3 + 0.3% w/v BSA) and subsequent exposure to H_2O_2 (Sigma, H1009). PRL or vehicle were also kept in the medium during H_2O_2 exposure. INS-1E cells exposed only to PRL were also used in the time course studies.

2.2 Assessment of cell viability. The cell viability was measure by: a) staining cells with DNA-binding dyes Hoechst 33342 (ThermoFisher, H3570; 10 μg/mL) and propidium iodide (ThermoFisher, P1304MP; 5 μg/mL). 100 uL of culture medium was removed from each well and added 100 uL of culture medium containing the DNA-binding dyes hoechst 33342 (ThermoFisher, H3570; 10 μg/mL) and propidium iodide (ThermoFisher, P1304MP; 5 μg/mL). After 15 min incubation, at 37 ºC, 100 uL of culture medium was removed from each well and added 100 uL of RPMI-1640 medium. The percentage of necrotic/apoptotic cells was determined on High Content Imaging System (ImageXpress; Molecular Devices) through the Live and Dead module of MetaXpress (Molecular Devices) software. In order to identify the viable and dead cell populations, masks were applied for the DAPI (350 nm excitation and 470 nm emission; Hoescht) and Texas Red (596 nm excitation and 615 nm emission; propidium iodide) wavelengths; b) western blot for cleaved caspase 3 (see details below).

2.3 Western blot assay. The cells were washed with PBS 1x and collected with 60 μL of protein extraction buffer Laemmli. The samples were heated at 100 ° C, for 5 min, for further application of 20 μL of their content on 12% acrylamide SDS-PAGE. After electrophoretic separation in apparatus, the proteins were transferred by liquid transfer with 20% methanol to nitrocellulose membrane (with a pore of 0.22 μm) and non-specific binding blockade was

performed with TBS-T buffer (50 mM Tris pH 7,5, 150 mM NaCl, 0,1% Tween®20) containing 10% skim milk, for 60 min. The membranes were incubated for 16 h, at 4 ° C, with primary antibodies to α-tubulin (Santa Cruz, 8095), β-actin (Santa Cruz, 81178), cleaved caspase 3 (abcam, 4051), CAT (abcam, 16731), GPx1 (abcam, 22604), SOD1 (abcam, 16831) and SOD2 (Santa Cruz, 30080). Next, the membranes were incubated for 60 min with appropriate horseradish peroxidase-conjugated secondary antibodies, at room temperature. Protein content was detected by ImageQuant LAS 4000 (Healthcare Bio-Sciences) after the chemiluminescence reaction with SuperSignal West Femto Chemiluminescent Substrate (Thermo Scientific). Finally, the densitometry of the protein bands was determined with ImageQuantTL (Healthcare Bio-Sciences) software. The values obtained were normalized by internal controls, α-tubulin or β-actin.

2.4 Real time quantitative PCR (qRT-PCR). Total RNA extraction was performed using TRIzol reagent (Invitrogen, 15596026), according to the manufacturer's instructions. Then, the RNA of the samples was quantified in a multimodal reader Spectramax i3 (Molecular Devices). The cDNA synthesis was done by means of the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, 4668814) and cDNA amplification was performed with the Power SYBR Green Master Mix reagent (Applied Biosystems, 4367659), along with the primers for *pparg* (Forward, 5´-CCTTGCTGTGGGGATGTCTC-3´; reverse, 3´ -CGCCAACAGCTTCTCCTTCT-5´) and to the housekeeping gene, *rps 26* (Forward, 5´-TTTTTCCTCCTTGGGCGTCTG-3´; reverse, 3´-GGTAGACAGTCGAATCATCCATTCA-5´). The qRT-PCR was done with the Applied Biosystems StepOne real-time PCR system (Applied Biosystems) and the mRNA expression was determined after normalization using the 2-ΔΔCT method [23].

2.5 MitoSOX. The cells were incubated for 20 min with mitoSOX probe (Invitrogen, M36008; 5 μM) and DNA-binding dye Hoechst 33342 (ThermoFisher, H3570; 5 μg/ml). MitoSOX is a mitochondrial O_2^- indicator, once undergoes oxidation by this ROS and is converted in a fluorescent product. The fluorescence acquisition was performed according to the manufacturer's instructions on a microplate reader using a High Content Imaging System (ImageXpress; Molecular Devices) and the module of analysis used was the Cell Scoring of MetaXpress software (Molecular Devices). Only the fluorescence of the mitoSOX in the cytoplasm was considered, since this probe migrates to the nucleus in cells in the process of apoptosis [24]. The values obtained were normalized by the number of

cells, determined by fluorescence of the Hoechst dye.

2.6 Mito-roGFP2-Orp1. INS-1E cells stably expressing mito-roGFP2-Orp1 were established by retroviral transduction. Briefly, Phoenix-AMPHO cells were transfected with pLPCX/mito-roGFP2-Orp1 (encoding the roGFP2-Orp1 probe with an N-terminal mitochondrial targeting sequence) by the calcium phosphate method. After 6 hours of transfection, cells were washed twice with PBS and transferred to fresh medium. After 24 and 48 hours of transfection, supernatant was collected and passed through a 0.22-mm filter. INS-1E cells (seeded at 2.7 × 105 cells per well in a six-well plate for 24 hours before each experiment) were incubated with the virus-containing supernatant supplemented with Polybrene (4 mg/mL) for 24 hours at 37°C in a 5% CO_2 atmosphere. Cells were then grown in medium containing puromycin (0.5 mg/mL) for several days. A highly fluorescent cell population (~6% of overall population) was selected by flow cytometry (FACSAria). Collected cells were expanded in the medium containing puromycin (0.5 mg/mL) and gentamycin (50 mg/mL). Pictures with a fluorescence microscope were taken to confirm the mitochondrial location of the probe. Mito-roGFP2-Orp1 is a H_2O_2-sensitive probe with two different and redox-dependent excitation peaks at 405 nm and 488 nm, and one single emission peak at 510 nm. While probe oxidation by H_2O_2 results in an increase of the 405 nm excitation peak, probe reduction by H_2O_2 increases excitation at 488 nm. Consequently, the ratio between 405/488 nm can be used as an indicator of the relative amount of oxidized/reduced mito-roGFP2-Orp1 and the H_2O_2 mitochondrial concentration [25]. After the pretreatment, the cells were exposed to H_2O_2 and immediatelly subjected to fluorescence reading in 405 and 480 nm during about 1h, by the CLARIOstar (BMG LABTECH). Fluorescences obtained 10 min after the addiction of H_2O_2, in a 10 min interval, were analyzed. The ratio of fluorescences (400/480) was normalized by the fluorescence ratio of the maximally-reduced and maximally-oxidized probe measured after addition of dithiothreitol (10 mM) and diamide (2 mM).

2.7 In silico assay. Transcriptome data were obtained from the macroarray of 1176 genes previously performed by our research group, using islets of Wistar rats (90 to 120 days of life; control) and islets of pregnant Wistar rats (15th day of pregnancy). Subsequent analyzes were performed with genes that presented relative differences above 2 fold change in relation to the control group and p value <0.05. These data were imported into the DAVID (Database for Annotation, Visualization and Integrated Discovery v6.8) software, in which evaluated

the enrichment of the KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways. It was also analyzed the enrichment of gene ontology by "cellular components" through GOterms-CC (http://www.geneontology.org).

2.8 Promoter reporter assay. The cells were transfected for 16h using RPMI 1640 medium, Opti-MEM medium (Gibco, 31985070) and Lipofectamine 3000 (Invitrogen, L3000015) with 0.5 μg of pRL-SV40 (luciferase renilla; Promega, E2231) and 30 ng of pPPRE X3-TK-luciferase (PPAR responsive element - PPRE; Dr. Bruce Spiegelman, Addgene, 1015) per well. After that, the transfection medium was replaced by the RPMI 1640 medium and after 24 h the cells were treated with vehicle or PRL (0.2 μg/mL, 0.5 μg/mL or 1.0 μg/mL) for that same period. The samples were collected with 100 μL of passive lysis buffer (Promega, E1500) and luciferase activity was measured in 20 μL of the lysate in a 96-well white plate (costar), using the Dual luciferase assay system (Promega) according to the manufacturer's instructions. Finally, the values obtained from the pPPRE X3-TK-luciferase activity were normalized by the renilla luciferase activity.

2.9 Small Interfering RNA (siRNA). Two different siRNAs against PPARγ (siPPARγ-1 and siPPARγ-2; Sigma-Aldrich) were used to knock down the expression of the target gene. Allstars Negative Control siRNA (Qiagen) was used as a negative control. The cells were incubated for 16h with RPMI 1640 medium, Opti-MEM medium and lipofectamine RNAiMAX (Invitrogen, 13778075) with 40 nM siPPARγ and 40 nM Allstars negative control siRNA (Negative control; Qiagen, 1027281). Next, the transfection medium was replaced by the RPMI 1640 medium and after 24 hours of recovery the treatment was performed.

2.10 Statistical Analysis. Results are presented as mean ± standard error of the mean (SEM). For the statistical evaluation was used t test and ANOVA (one or two way), followed by Bonferroni posttest. The level of significance was set at $p < 0.05$ and the analyzes were done through the GraphPad Prism 6.00 software. The number of samples used is described in the graphics legend. In the in silico method the cut-off values used for KEGG pathway enrichment analysis were $p < 0.1$ and Fold enrichment $p \geq 1.5$ [26]. The results of this analysis are presented in -log Fold Change.

3. Results

3.1 PRL prevents the cell death induced by H_2O_2. Firstly, we tested if PRL could increase the survival of INS-1E cells against oxidative stress. The treatment with PRL reduced the protein content of cleaved caspase 3 by 42% in the presence of 100 μM and 200 μM of H_2O_2 (Fig. 1A) and in 82% and 42% the percentage of dead cells compared to 100 μM and 150 μM H_2O_2, respectively (Fig. 1B).

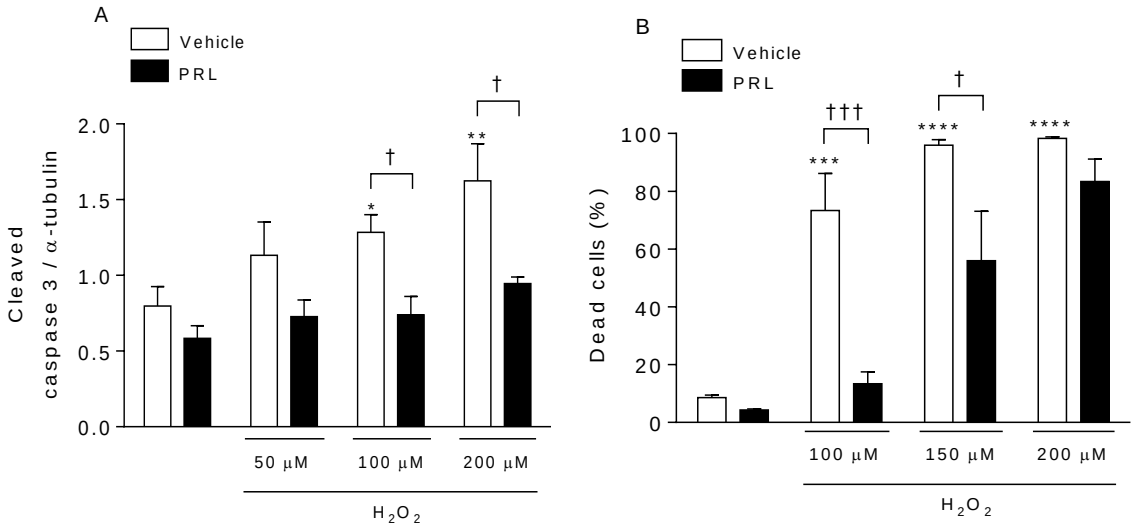
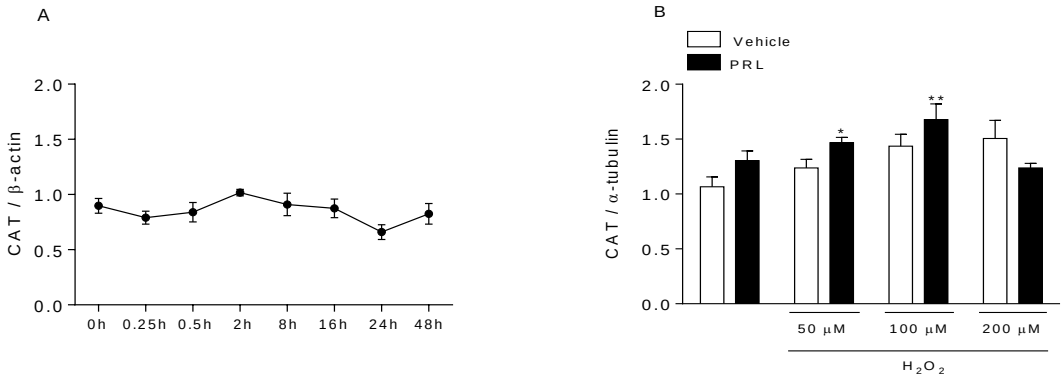
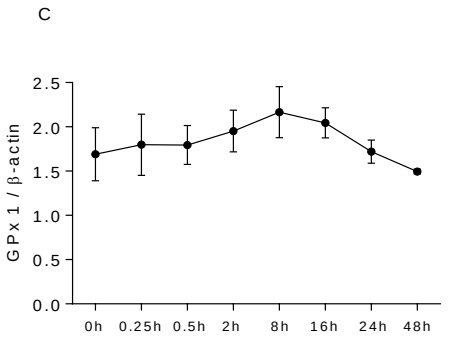
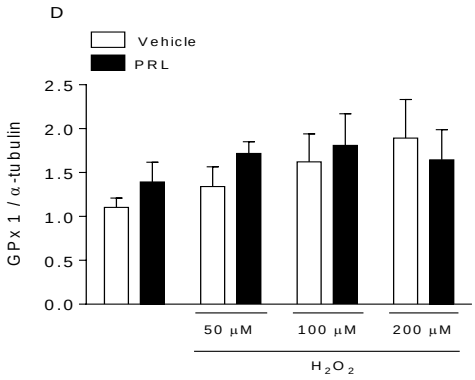


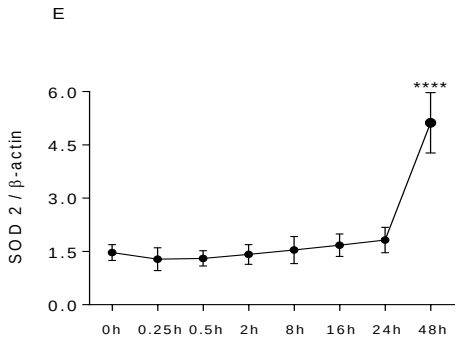
Figure 1. Viability of cells treated with vehicle or PRL and oxidative stress inducer. INS-1E cells were pretreated for 24h with vehicle or PRL. After this period, the oxidative stress induction was done adding different concentrations of H_2O_2 for another 24 hours. (A-B) Protein content of the cleaved caspase 3 determined by western blotting. The representative image is in Fig 2I; (C) Percentage of dead cells using the dyes Hoescht 33342 and propidium iodide. The results represent the means ± SEM. Comparisons were performed by two-way ANOVA followed by the Bonferroni posttest. *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 versus vehicle; † p<0.05, ††† p<0.001 between the bars (n = 4).

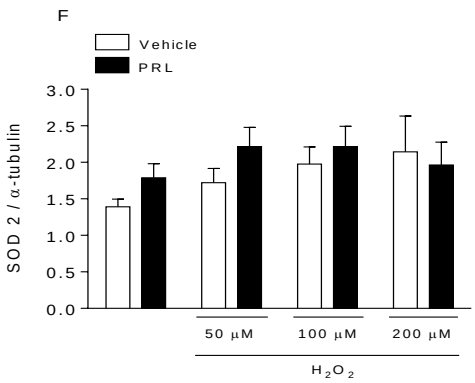
3.2 PRL increases the protein content of antioxidant enzymes. We investigated if PRL could modulate the protein content of antioxidant enzymes for the prevention of cell death caused by H_2O_2. In the time course of PRL, we observed an increase in the protein content of SOD2 with 48h, but not of CAT, GPx1, SOD1 (Fig. 2A, 2C, 2E and 2G). Interestingly, when the cells were pretreated with PRL and treated with H_2O_2 (50 μM and 100 μM), we observed an increase in CAT expression, but not in the other antioxidant enzymes (Fig. 2B, 2D e 2F).

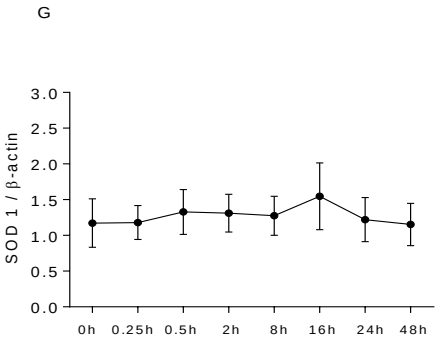


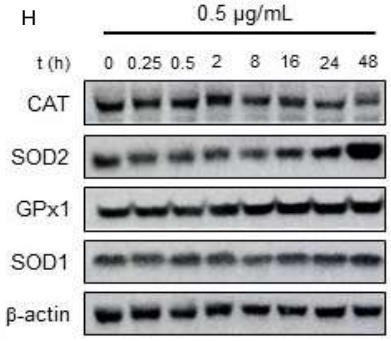

C
GPx 1 / β-actin
2.5
2.0
1.5
1.0
0.5
0.0
0h 0.25h 0.5h 2h 8h 16h 24h 48h


D
Vehicle
PRL
GPx 1 / α-tubulin
2.5
2.0
1.5
1.0
0.5
0.0
50 μM
100 μM
200 μM
H2O2


E
SOD 2 / β-actin
****
6.0
4.5
3.0
1.5
0.0
0h 0.25h 0.5h 2h 8h 16h 24h 48h


F
Vehicle
PRL
SOD 2 / α-tubulin
3.0
2.5
2.0
1.5
1.0
0.5
0.0
50 μM
100 μM
200 μM
H2O2


G
SOD 1 / β-actin
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0h 0.25h 0.5h 2h 8h 16h 24h 48h


H
0.5 μg/mL
t (h)
0 0.25 0.5 2 8 16 24 48
CAT
SOD2
GPx1
SOD1
β-actin

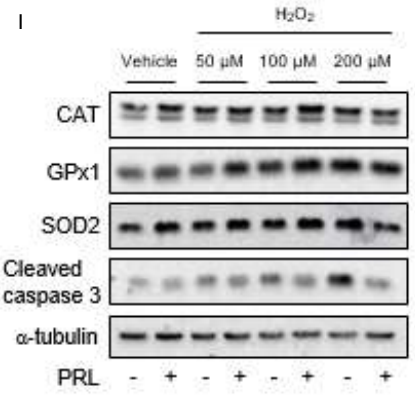
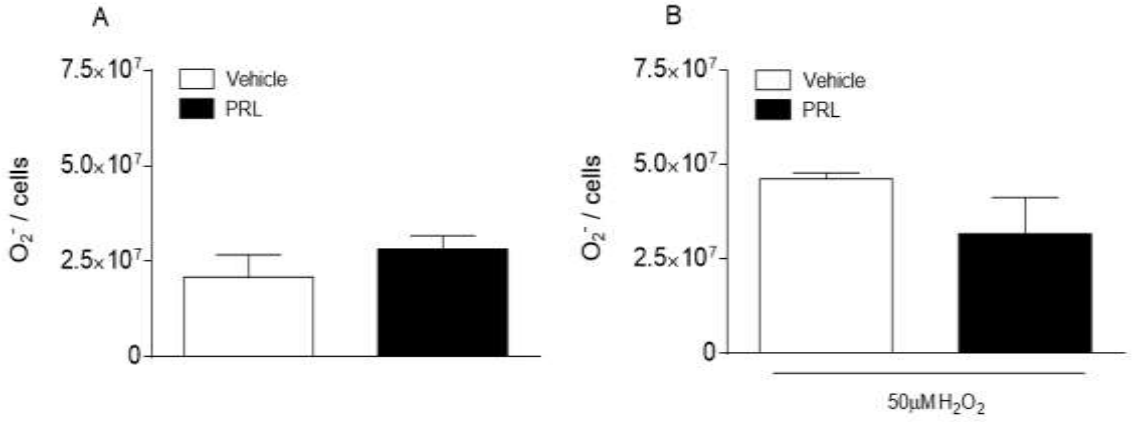


Figure 2. Protein content of CAT, GPx1, SOD2 and SOD1 in cells treated with vehicle or PRL and oxidative stress inducer. (A, C, E, G and H) INS-1E cells were treated with PRL (0.5 µg/mL) for 0h, 0.25h, 0.5h, 2h, 8h, 16h, 24h, and 48h. For the statistical evaluation, one-way ANOVA was used, followed by the Bonferroni posttest. (B, D, F and I) INS-1E cells were pretreated for 24h with vehicle or PRL. After this period, the oxidative stress induction was done adding different concentrations of H_2O_2 for another 24 hours. Comparisons were performed by two-way ANOVA followed by the Bonferroni posttest. Protein content of antioxidant enzymes was determined by western blotting and the results represent the means ± SEM. **p<0.01 versus vehicle, ****p < 0.0001 versus t=0h (n = 4-5).

3.3 PRL prevents the increase of the mitochondrial concentration of H_2O_2. As PRL seems to influence the expression of antioxidant enzymes, we used the MitoSOX and mito-GFP2-ORP1 probes to investigate whether treatment with the hormone prevent the increase in ROS content induced by 50 µM of H_2O_2 (Appendix, Fig 1A and B). We did not find effect of PRL on the mitochondrial O_2^- concentration both in the basal state (Fig. 3A), and in cells exposed to H_2O_2 (Fig. 3B). However, we observed that PRL prevent the oxidation by H_2O_2 in the mitochondria, that is, reduced the mitochondrial concentration of H_2O_2, in the basal state (Fig. 3C) and after the addition of H_2O_2 (Fig. 3D).



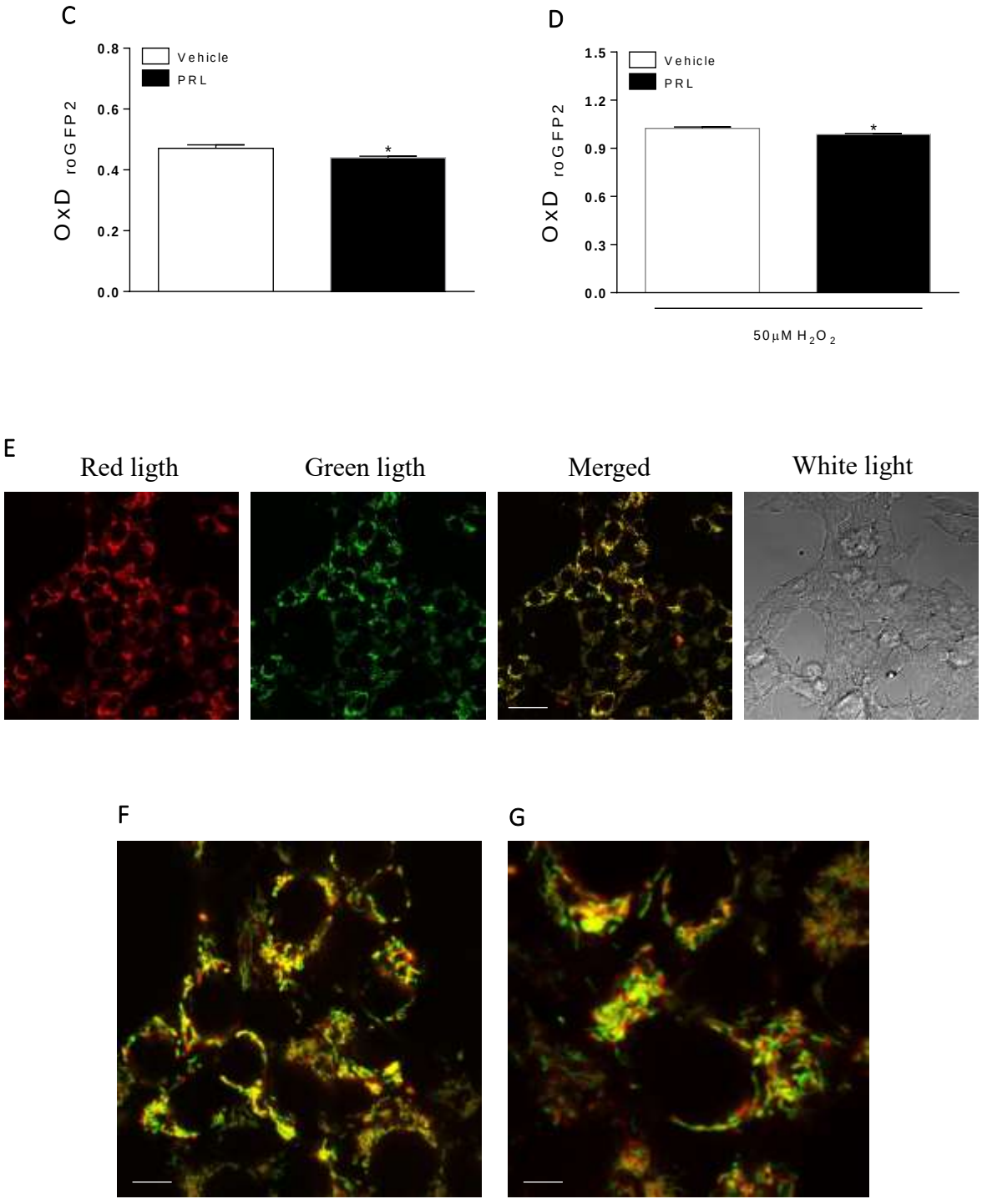


Figure 3. Production of O_2^- and H_2O_2 mitochondrial. INS-1E cells were pretreated for 24h with vehicle or PRL. After, the oxidative stress was induced with (B) 50 µM of H_2O_2 for another 6 hours and (D) 50 µM of H_2O_2, while reading the plate. (A and B) Mitochondrial O_2^- detection was performed with the mitoSOX probe, 5 µM, with reading done in High-Content Imaging System. The values obtained were normalized by the number of cells, determined by using Hoechst 33342 DNA dye (10 µg/mL). Comparisons were performed t test (n = 3). (C and D) It was performed the fluorescence reading in 400 and 480 nm during about 1h, by the the CLARIOstar (BMG LABTECH). Fluorescences obtained 10 min after the addiction of H_2O_2, in a 10 min interval, were analyzed. The ratio of fluorescences (400/480) was normalized to the fluorescence ratio of the maximally-reduced and maximally-oxidized probe measured after addition of 10 mmol/L dithiothreitol and 100 mmol/L diamide. Comparisons were performed by t test. The results represent the means ± SEM (n = 5).

**p < 0.01, ****p < 0.0001 versus vehicle. (E) mito-GFP2-ORP1 fluorescences with red light (405 nm; oxidized state of the probe), green light (488 nm; reduced state of the probe) and merged, confirming the mitochondrial location of probe. Scale bar: 40 µm. (F and G) merged in details. Scale bar: 60 µm and 100 µm, respectively.

3.4 PPAR signaling is enriched in islets of pregnant rats. The genes upregulated in the islets of pregnant rats previously identified from macroarray by our research group, were transferred to DAVID. This software grouped the genes according to the pathway that they are related to and caught our attention the enrichment of the PPAR signaling pathway (Fig. 4; Appendix, table 2), which is involved in the production of antioxidant enzymes in different cell types, including beta cells [27-29].

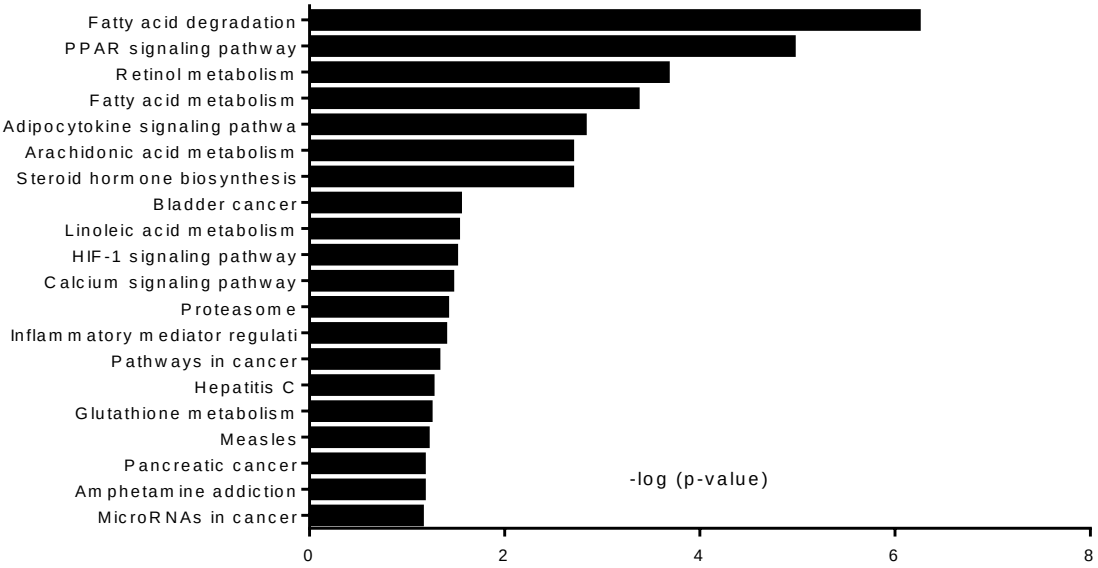


Figure 4. Enrichment of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways in islets of pregnant rats. The upregulated genes in islets from pregnant rats were imported into the DAVID software that grouped the genes according to the cell signaling pathways available in KEGG. The results are presented in -log Fold Change.

The ontological analysis of these same genes by "cellular components" revealed great influence of pregnancy on the expression of mitochondrial genes (Appendix, table 3) since 16 genes were upregulated in this organelle (Appendix, table 4). In addition, five of these genes are associated with both the fatty acid degradation pathway and PPAR signaling, they are: acyl-CoA synthetase (Acs), responsible for the mitochondrial transport of fatty acids; acyl-CoA oxidase (Aco), carnitine palmitoyltransferase 1 (Cpt-1), carnitine palmitoyltransferase 2 (Cpt-2) and acyl-CoA dehydrogenase (Lcad), related to the oxidation of fatty acids (Appendix, fig. 3).

3.5 PRL actives PPAR, however PPARγ knockdown does not decreases the beneficial effect of PRL on cell viability. Based on the results of the analysis in silico, we investigated if PRL modulates the activity of PPARs in beta cells. For this purpose, we performed the promoter reporter assay was performed targeting PPAR response element (PPRE), which are specific sequences in the promoter region of genes were PPARs bind to start its transcription. Interestingly, the PRL (0.5 μg/ml and 1.0 μg/mL) promoted PPRE activation (Fig. 5A), indicating that this hormone activates PPARs.

Among the PPAR isoforms, PPARγ is the most extensively studied in beta cells. Furthermore, PRL increases the expression of PPARγ in adipocyte cell line and rat adipose tissue [30]. Therefore, we tested whether PPARγ is involved in the protective action of PRL against the oxidative stress in INS-1E cells. For this, two specific siRNAs (siPPARγ-1 and siPPARγ-2) were used to suppress PPARγ in INS-1E cells. Both siRNA inhibited by, approximately, 55% the expression of PPARγ (Fig 5B). However, this inhibition did not abrogate the efficiency of PRL in preventing the cell death induced by H_2O_2 (Fig 5C).

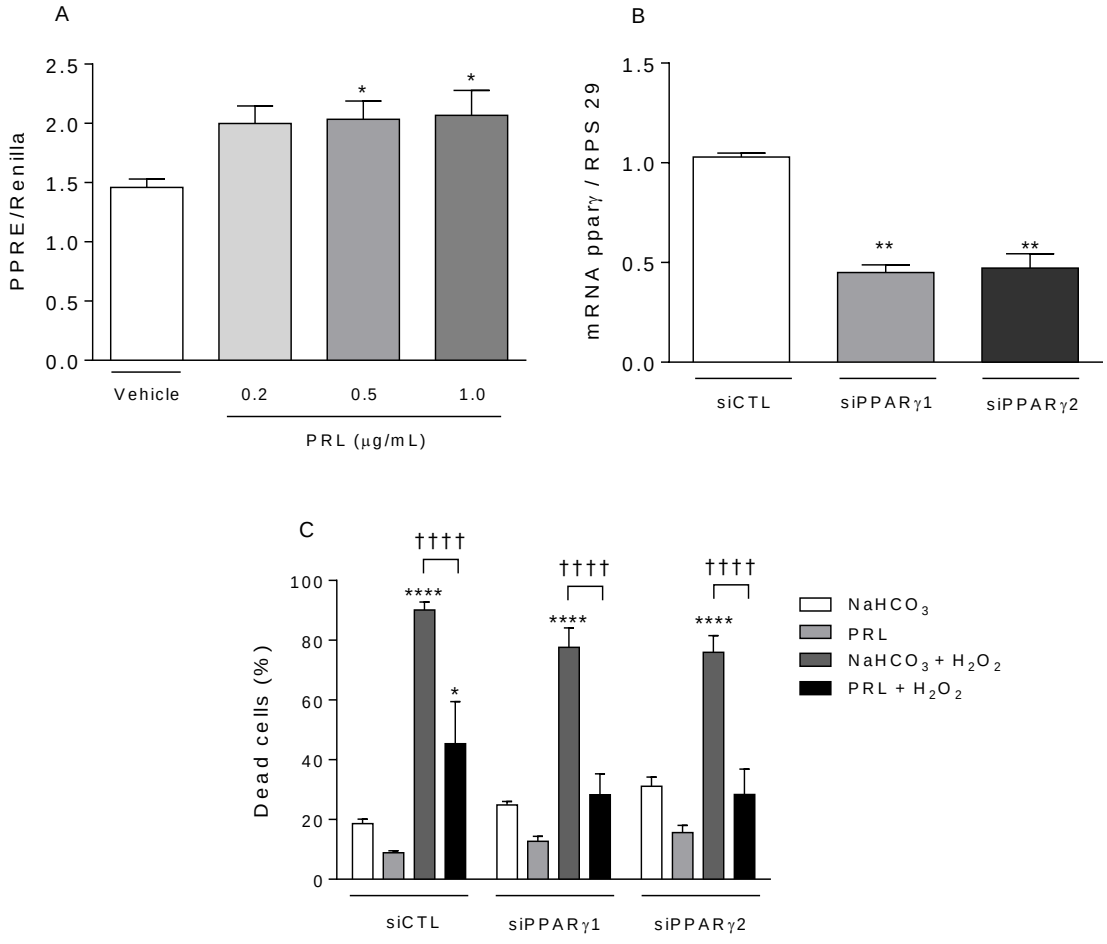


Figure 5. Activation of PPRE by PRL and viability of cells treated or not with PRL and oxidative stress inducer, after PPARγ knockdown. (A) INS-1E cells were transfected for 16 h with 0.5 μg renilla luciferase and 30 ng pPPRE X3-TK-luciferase per well. After this time the transfection medium was changed to the RPMI 1640, where the cells remained for 24 hours. Then the cells were treated with 0.2 μg/mL, 0.5 μg/mL and 1.0 μg/mL of PRL for 24 hours. The luciferase activity was measured in 20 μL of lysate using the Dual luciferase assay system. Finally, the values obtained from the pPPRE X3-TK-luciferase activity were normalized by the renilla luciferase values. The results represent the means ± SEM. Comparisons were performed by one-way ANOVA followed by the Bonferroni posttest. *p<0.05 versus vehicle (n=4). (B) INS-1E cells were transfected for 16 h with 40 nM of two specifics siRNAs for PPARγ (siPPARγ1 and siPPARγ2) or a control siRNA (siCTL). After, the transfection medium was exchanged for RPMI 1640, where the cells remained for 72 hours before being collected. Gene expression was determined qRT-PCR, normalized by the 2^-ΔΔCt method (n = 2). (C) INS-1E cells were transfected for 16 h with 40 nM of two specifics siRNAs to PPARγ (siPPARγ1 and siPPARγ2) or a control siRNA (siCTL). 48h after transfection, the cells were pretreated for 24h with vehicle or PRL and the oxidative stress induction was gone adding 100 μM of H_2O_2 for another 24h. The percentage of dead cells was determined using the dyes Hoescht 33342 and propidium iodide. The results represent the means ± SEM. Comparisons were performed by two-way ANOVA followed by the Bonferroni posttest. *p<0.05 and ****p<0.0001 versus vehicle; †††† p<0.0001 between the bars (n = 4).

4. Discussion

DM is related, mainly, with the modern life style, unhealthy diet and sedentary life [31], and has been affecting more and more people over time. The severity of the disease is due to the complications that untreated hyperglycemia can cause as: susceptibility to infections, retinopathy with possible vision loss, nephropathy inducing kidney failure, peripheral neuropathy related with the presence of foot lesions, atherosclerosis and cerebrovascular diseases [32]. Taking into account all these injuries that DM can cause, it becomes imperative to investigate molecules and mechanisms that inhibit or even prevent the development of this disease.

Our study demonstrated for the first time that PRL increases the expression of SOD2 and CAT, decreases the mitochondrial concentration of H_2O_2 and is able to activate PPAR in INS-1E cells. Notably, in oxidative stress condition, PRL increased the protein content of CAT after 24h. This enzyme detoxifies H_2O_2 [33] and has an important relationship with the development of diabetes, because knockout mice for the enzyme and acatalasemic mice present pre-diabetic phenotype [34] and are more susceptible to diabetes caused by alloxan [35], respectively. There are controversies regarding the profile of antioxidant enzymes in different tissues in DM but often they are impaired, certainly because hyperglycemia enhances non-enzymatic binding of glucose to proteins (glycation), causing structural and functional changes [36] and alters the availability of micronutrients that serve as cofactors for antioxidant enzymes [37]. Therefore, approaches that favor the production of antioxidant enzymes are important to beta cell protection and functioning of the beta cell.

Our next step was to evaluate whether PRL could reduce ROS content of the cells. Using a specific mitochondrial O_2^- probe, the mitoSOX, we did not observe changes in the

mitochondrial O_2^- levels in INS-1E cells with PRL-pretreatment. As previously mentioned, the increase in the catalase protein content occurred 24 hours after stress induction by H_2O_2. It is possible that at 6h the increase in CAT synthesis has not yet occurred and, thus, the O_2^- mitochondrial concentration was not altered. In this sense, the enzymatic activity assay can be used to clarify the behavior of the enzyme, regardless of its protein content. Interestingly, using a specific probe for H_2O_2, the mito-roGFP2-ORP1, we observed that PRL prevented the probe oxidation, both in the basal state and after the addition of H_2O_2, at the time of reading. Likewise, the expression of CAT has not yet increased, suggesting the participation of another detoxification pathway of H_2O_2. Another enzyme that detoxifies H_2O_2 is peroxiredoxin and studies have shown its importance for protection of beta cell against death induced by oxidative stress [38, 39].

An experimental approach that has gained increasing prominence in the scientific field is the in silico analysis. Through this bioinformatics tool it is possible to use information available in databases to list research targets, such as genes. Our bioinformatics analysis made from the genes upregulated during pregnancy, a condition in which there is an increase in the hypothalamic secretion of PRL [12], showed the enrichment of transcription factors involved with the production of antioxidant enzymes, the PPARs. This transcription factors are present in three isoforms: α, β/δ and γ. PPARs form a heterodimer with the retinoid x receptor (RXR) that binds to specific sequences in the promoter region of the target genes, the PPRE, promoting changes on chromatin structure and activation of transcription [40]. The promoters of the genes of antioxidant enzymes have PPRE [27,29,41] and the activation of PPAR increases expression and activity of antioxidant enzymes such as CAT [27-29] and SOD [28, 42].

In addition to the enrichment of PPAR pathway, it was remarkable the large number of upregulated genes located in the mitochondria and involved with the metabolism of fatty acids in pancreatic islets during pregnancy. As an important function of PPARs is the control of lipid metabolism [43,44], these results suggest that, in pregnancy, the PPAR pathway is enriched to promote the metabolism of fatty acids [45,46]. In fact, the concentration of circulating lipids increases during this period, as a result, the islets adapt to become more sensitive to fatty acids [47]. Fatty acids metabolism by beta cells, as well as glucose metabolism, increases the ATP/ADP ratio, promoting increased insulin secretion [48,49]. This increase in insulin secretion acts as a compensatory mechanism to resistance to this hormone that is verified during pregnancy [14]. At the same time, the increase in fatty acid metabolism results in more NADH and $FADH_2$ available to the electron transport chain,

adding the production of ATP and also of ROS [50]. It would be possible that, to neutralize the increase of ROS, the PPAR pathway also would act to increase the production of antioxidant enzymes in order to protect beta cells from oxidative stress. That is, alterations in the metabolism and redox state of beta cells could be modulated by a common pathway involving PPARs (Fig 8).

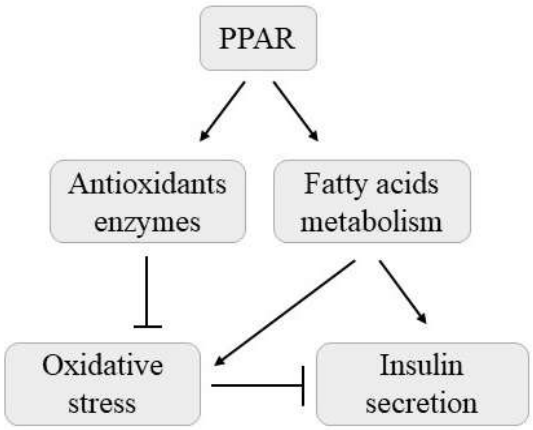


Figure 6. Possible involvement of PPARs pathway in the metabolism and redox state of beta cells.

To test whether PRL has influence on PPARs, as suggested by in silico analyzes, we performed the promoter reporter assay and found that PRL activates these transcription factors. Among PPARs, PPARγ is the therapeutic target of drugs indicated for the treatment of diabetes, thiazolidinediones (TZDs), such as pioglitazone, for example [51,52]. In diabetic *db/db* and obese pre-diabetic animal models, TZDs increase proliferation and reduce apoptosis of beta cells [53,54] and stimulate insulin secretion [53,55], respectively. Faced with oxidative stress, pioglitazone prevent the formation of ROS protecting pancreatic islets and beta cells from apoptosis [54,56]. In this sense, Kanda et al. 2010 and Chung at al. 2011 demonstrated that TZDs increase expression of CAT, GPx and SOD2, both in islets and beta cells.

Because of the actions that the activation of PPARγ exerts on the insulin producing cells and knowing that the PRL increases the expression of this isoform of PPAR in adipocyte cell line and rat adipose tissue [30], we evaluated whether PRL is PPARγ-dependent to protect INS-1E cells against oxidative stress. Despite the knockdown of PPARγ, PRL maintained its beneficial effect on cell viability against H_2O_2-induced death. It is possible that another PPAR isoforms play an important role in the viability, since they are also present in beta cells [57], or that other pathways may be involved, such as the classic pathway of antioxidant enzymes production by the transcription factor nuclear factor

erythroid 2–related factor 2 (Nrf-2) which protects beta cells from oxidative damage [58,59]. Another mechanism that deserves to be highlighted is that involves c-Jun N-terminal kinase (JNK) signaling. JNK is activated by ROS to induce cell death by means of phosphorylation of pro-apoptotic proteins [60,61]. In contrast, PRL negatively regulates JNK by preventing the beta cells death by inflammatory cytokines [19] that, like ROS, activate JNK to lead to cell death [62,63].

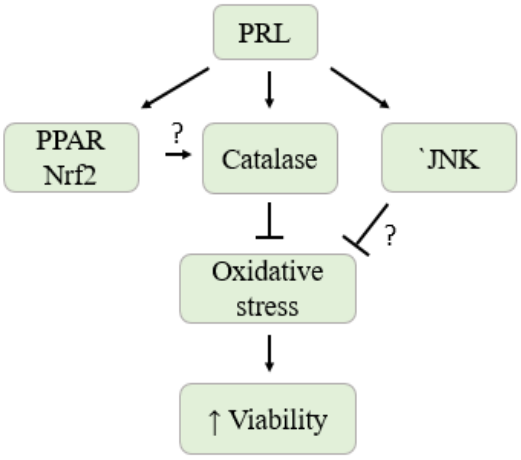


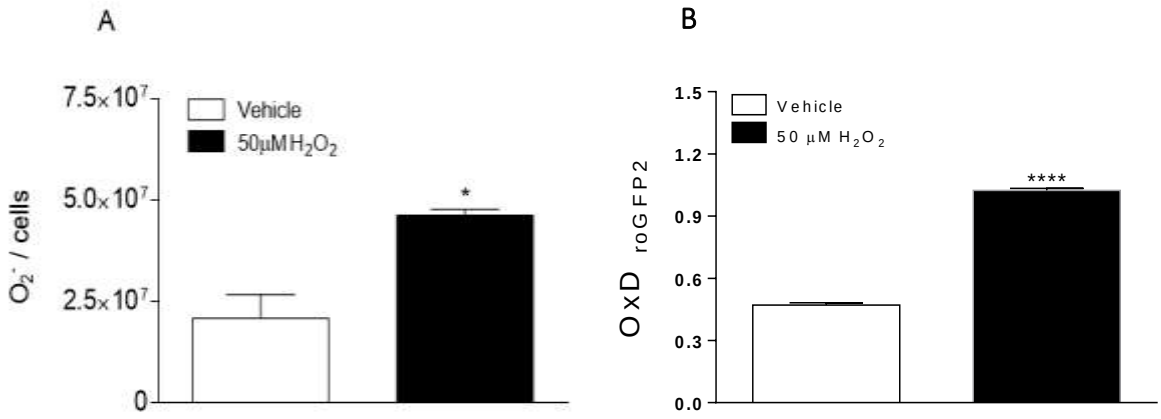
Figure 7. Mechanism that can be used by PRL to protect beta cells against oxidative stress.

In conclusion, we demonstrate for the first time that PRL is capable of activating PPAR. However, PRL is not dependent on PPARγ to protect INS-1E cells from H_2O_2-induced death, therefore future researches are needed to reveal the exacts mechanisms of beta cell protection by PRL against oxidative stress. In addition, we also revealed that PRL increases the expression of antioxidant enzymes and decreases the mitochondrial concentration of H_2O_2, important effects in favor of pancreatic beta cell survival (Fig 9).

5. Acknowledgements

This work was supported by grants from the São Paulo Research Foundation (FAPESP; grant number 2015/12611-0), National Counsel of Technological and Scientific Development (CNPq) and Coordination for the Improvement of Higher Education Personnel (CAPES; grant number 17693/2015), Brazil. We thank Rodrigues G. and Moraes S.S. for technical assistance.

6. Appendix



Supplementary figure 1. Production of O_2^- and H_2O_2 mitochondrial. INS-1E cells were pretreated for 24h with vehicle. After, the oxidative stress was induced with (A) 50 µM of H_2O_2 for another 6 hours and (B) 50 µM of H_2O_2, while reading the plate. (A) Mitochondrial O_2^- detection was performed with the mitoSOX probe, 5 µM, with reading done in High-Content Imaging System. The values obtained were normalized by the number of cells, determined by using hoechst 33342 DNA dye (10 µg/mL). Comparisons were performed t test (n = 3). (B) It was performed the fluorescence reading in 400 and 480 nm during about 1h, by the the CLARIOstar (BMG LABTECH). Fluorescences obtained 10 min after the addiction of H_2O_2, in a 10 min interval, were analyzed. The ratio of fluorescences (400/480) was normalized to the fluorescence ratio of the maximally-reduced and maximally-oxidized probe measured after addition of 10 mmol/L dithiothreitol and 100 mmol/L diamide. Comparisons were performed by t test. The results represent the means ± SEM (n = 5). *p < 0.05, ****p < 0.0001 versus vehicle.

Supplementary table 1. Enrichment of KEGG pathway in islets of pregnant rats.

Term	Count	%	PValue
Fatty acid degradation	7	8.75	5.77E-07
PPAR signaling pathway	7	8.75	1.09E-05
Retinol metabolism	6	7.5	2.15E-04
Fatty acid metabolism	5	6.25	4.38E-04
Adipocytokine signaling pathway	5	6.25	1.52E-03
Arachidonic acid metabolism	5	6.25	2.02E-03
Steroid hormone biosynthesis	5	6.25	2.02E-03
Bladder cancer	3	3.75	2.91E-02
Linoleic acid metabolism	3	3.75	3.04E-02
HIF-1 signaling pathway	4	5	3.20E-02
Calcium signaling pathway	5	6.25	3.46E-02
Proteasome	3	3.75	3.91E-02
Inflammatory mediator regulation of TRP channels	4	5	4.12E-02
Pathways in cancer	7	8.75	4.82E-02
Hepatitis C	4	5	5.47E-02
Glutathione metabolism	3	3.75	5.71E-02
Measles	4	5	6.10E-02
Pancreatic cancer	3	3.75	6.79E-02
Amphetamine addiction	3	3.75	6.79E-02
MicroRNAs in cancer	4	5	7.00E-02

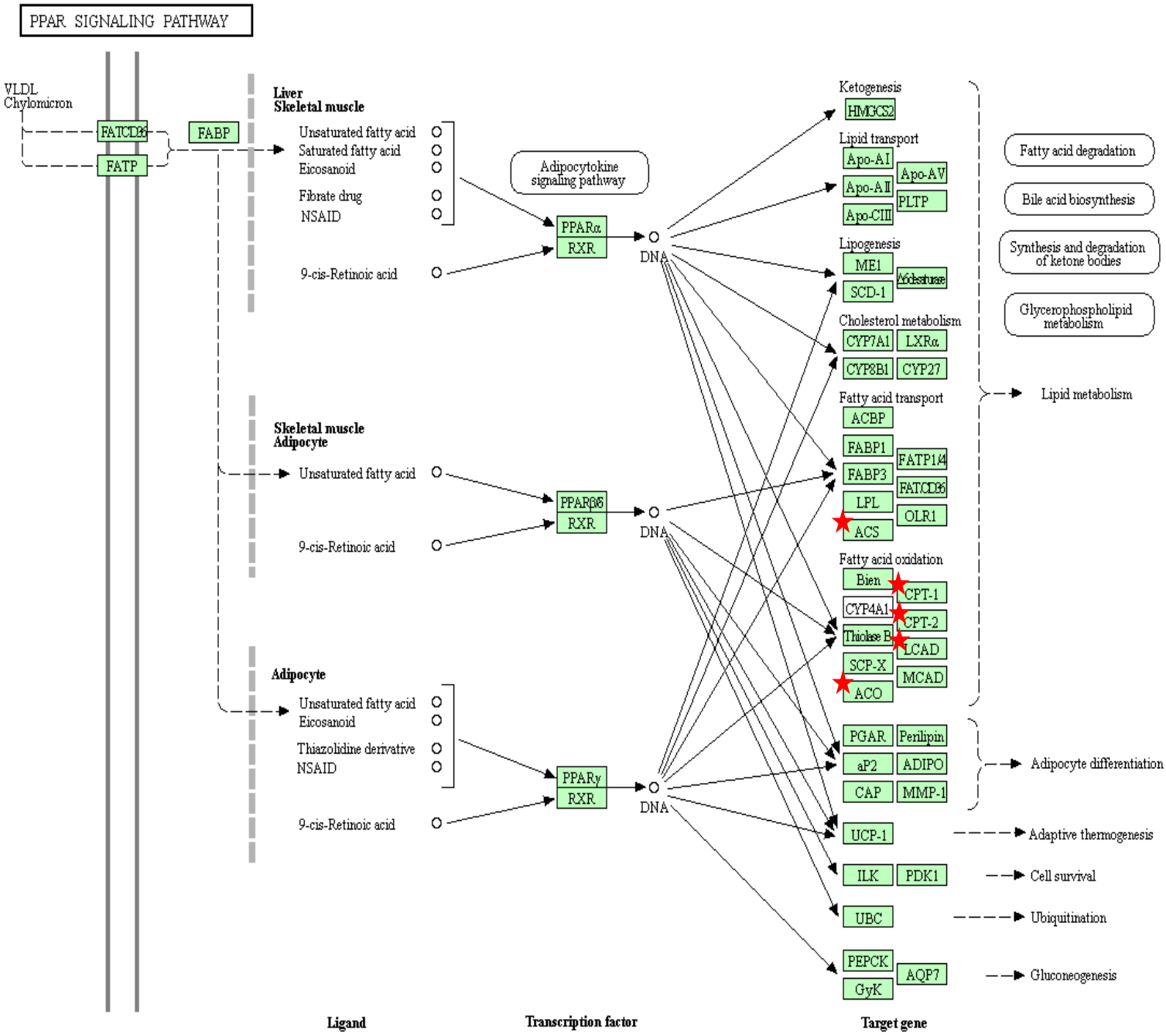
Supplementary table 2. Cellular components in descending order of the number of genes upregulated in the islets of pregnant rats.

Term	Count	%	PValue
Organelle membrane	7	8.75	8.75E-07
Endoplasmic reticulum membrane	11	13.75	2.92E-05
Secretory granule	6	7.5	4.07E-05
Membrane	21	26.25	6.54E-05
Intracellular membrane-bounded organelle	11	13.75	1.72E-04
Proteasome core complex, alpha-subunit complex	3	3.75	4.57E-04
Mitochondrion	16	20	4.93E-04
Intracellular organelle	3	3.75	1.14E-03
Presynaptic membrane	4	5	2.44E-03
Proteasome core complex	3	3.75	2.59E-03
Vesicle	5	6.25	2.76E-03
Apical plasma membrane	6	7.5	5.49E-03
Cell surface	8	10	6.51E-03
Extracellular space	12	15	7.23E-03
Synaptic vesicle	4	5	1.14E-02
Neuron projection	6	7.5	1.38E-02
Cell junction	6	7.5	1.63E-02
Synaptic vesicle membrane	3	3.75	1.93E-02
Proteasome complex	3	3.75	1.93E-02
Cytoplasm	28	35	2.20E-02
Extrinsic component of cytoplasmic side of plasma membrane	3	3.75	2.52E-02
Mitochondrial inner membrane	5	6.25	2.67E-02
Plasma membrane	22	27.5	3.71E-02
Basolateral plasma membrane	4	5	3.91E-02
Extracellular exosome	16	20	4.84E-02
Dense core granule	2	2.5	4.95E-02
Postsynaptic density	4	5	5.51E-02

Focal adhesion	5	6.25	5.52E-02
Z disc	3	3.75	6.71E-02
Protein complex	6	7.5	7.00E-02
Perinuclear region of cytoplasm	6	7.5	8.25E-02
Dendrite	5	6.25	9.64E-02

Supplementary table 3. Mitochondrial genes upregulated in the islets of pregnant rats.

ID	Gene Name
Lcad	acyl-CoA dehydrogenase, long chain
Acox1	acyl-CoA oxidase 1
Acsl6	acyl-CoA synthetase long-chain family member 6
Ak3	adenylate kinase 3
Cpt1a	carnitine palmitoyltransferase 1A
Cpt2	carnitine palmitoyltransferase 2
Cox1	cytochrome c oxidase subunit 1
Gsr	glutathione-disulfide reductase
Ghr	growth hormone receptor
Hsd3b1	hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1
Lypla1	lysophospholipase I
Mmp2	matrix metallopeptidase 2
Mmp3	matrix metallopeptidase 3
Nr3c1	nuclear receptor subfamily 3, group C, member 1
Stat3	signal transducer and activator of transcription 3
Slc16a1	solute carrier family 16 member 1



Supplementary figure 2. PPAR signaling pathway. The pregnant rats islets present upregulation of five genes (star) regulated by PPAR and related to degradation of fatty acids. Source: DAVID, 2018.

7. References

[1] WHO. World Health Organization. Diabetes. Available at: http://www.who.int/diabetes/en/. Accessed on: 10 nov. 2018.

[2] H.E. Thomas, M.D. McKenzie, E. Angstetra, P.D. Campbell, T.W. Kay, Beta cell apoptosis in diabetes, Apoptosis. 14 (2009) 1389–1404.

[3] R.A. Defronzo, E. Ferrannini, L. Groop, R.R. Henry, W.H. Herman, J.J. Holst, F.B. Hu, C.R. Kahn, I. Raz, G.I. Shulman, D.C. Simonson, M.A. Testa, R. Weiss, Type 2 diabetes mellitus, Nat. Rev. 1 (2015) 1–23.

[4] H. Sies, D. Jones, Oxidative Stress, Encycl. Stress. 3 (2007) 45–48.

[5] A. Ceriello, Oxidative stress and glycemic regulation, Metabolism. 49 (2000) 27–29.

[6] D. Lichtenberg, I. Pinchuk, Oxidative stress, the term and the concept, Biochem. Biophys. Res. Commun. 23 (2015) 1–4.

[7] P. Newsholme, E.P. Haber, S.M. Hirabara, E.L.O. Rebelato, J. Procopio, D. Morgan, H.C. Oliveira-Emilio, A.R. Carpinelli, R. Curi, Diabetes associated cell stress and dysfunction: role of mitochondrial and non-mitochondrial ROS production and activity, J. Physiol. 583 (2007) 9–24.

[8] F.A. Matough, S.B. Budin, Z.A. Hamid, N. Alwahaibi, J. Mohamed, The Role of Oxidative Stress and Antioxidants in Diabetic Complications, Med. J. 12 (2012) 5–18.

[9] M. Valko, D. Leibfritz, J. Moncol, M.T.D. Cronin, M. Mazur, J. Telser, Free radicals and antioxidants in normal physiological functions and human disease, Int. J. Biochem. Cell Biol. 39 (2007) 44–84.

[10] M.E. Freeman, B. Kanyicska, A. Lerant, G. Nagy, Prolactin: Structure, Function, and Regulation of Secretion, Physiol. Rev. 80 (2000) 1523–1631.

[11] A. Ignacak, M. Kasztelnik, T. Sliwa, R.A. Korbut, K. Rajda, T.J. Guzik, Prolactin - Not only lactotrophin a “new” view of the “old” hormone, J. Physiol. Pharmacol. 63 (2012) 435–443.

[12] A.G. Frantz, Prolactin, Physiol. Med. 298 (1978) 201–207.

[13] T.C. Brelje, D.W. Scharp, P.E. Lacy, F. Talamantes, L. Ogren, M. Robertson, H.. Friesen, R.L. Sorenson, Effect of homologous placental lactogens, prolactins, and growth hormones on islet B-cell division and insulin secretion in rat, mouse, and human islets: implication for placental lactogen regulation of islet function during pregnancy, Endocrinology. 132 (1993) 879–887.

[14] R.L. Sorenson, T.C. Brelje, Adaptation of Islets of Langerhans to Pregnancy: 3-Cell Growth, Enhanced Insulin Secretion and the Role of Lactogenic Hormones, Horm. Metab. Res. 29 (1997) 301–307.

[15] M. Freemark, I. Avril, D.O.N. Fleenor, P. Driscoll, A.N.N. Petro, E. Opara, W. Kendall, J.O.N. Oden, S. Bridges, N. Binart, B. Breant, P.A. Kelly, Targeted deletion of the PRL receptor: Effects on islet development, insulin production, and glucose tolerance, Endocrinology. 143 (2002) 1378–1385.

[16] T.C. Brelje, J.A. Parsons, R.L. Sorenson, Regulation of Islet p-Cell Proliferation by Prolactin in Rat Islets, Diabetes. 43 (1994) 263–273.

[17] A.C. Boschero, C.S. Crepaldi, M.E. Carneiro, E. Delattre, I. Atwater, Prolactin Induces Maturation of Glucose Sensing Mechanisms in Cultured Neonatal Rat Islets, Endocrinology. 133 (1992) 515–520.

[18] T. Yamamoto, A. Mita, C. Ricordi, S. Messinger, A. Miki, Y. Sakuma, F. Timoneri, S. Barker, A. Fornoni, R.D. Molano, L. Inverardi, A. Pileggi, H. Ichii, Prolactin Supplementation to Culture Medium Improves β-Cell Survival, Transplantation. 89 (2010) 1328–1335.

[19] T.R. Nardelli, E.C. Vanzela, K.C. Benedicto, F. Brozzi, A. Fujita, A.K. Cardozo, D.L. Eizirik, A.C. Boschero, F. Ortis., Prolactin protects against cytokine-induced beta cell death by NFkB and JNK inhibition, J. Mol. Endocrinol. 61 (2018) 25–36.

[20] S. Bordin, M.E.C. Amaral, G.F. Anhê, V. Delghingaro-Augusto, D.A. Cunha, J.E. Nicoletti-Carvalho, A.C. Boschero, Prolactin-modulated gene expression profiles in pancreatic islets from adult female rats, Mol. Cell. Endocrinol. 220 (2004) 41–50.

[21] T. Adachi, M. Inoue, H. Hara, S. Suzuki, Effects of PPARgamma ligands and C/EBPbeta enhancer on expression of extracellular-superoxide dismutase., Redox Rep. 9 (2004) 207–

212.
[22] R. Meléndez García, D. Arredondo Zamarripa, E. Arnold, X. Ruiz-Herrera, R. Noguez Imm, G. Baeza Cruz, N. Adán, N. Binart, J. Riesgo-Escovar, V. Goffin, B. Ordaz, F. Peña-Ortega, A. Martínez-Torres, C. Clapp, S. Thebault, Prolactin protects retinal pigment epithelium by inhibiting sirtuin 2-dependent cell death, EBioMedicine. 7 (2016) 35–49.
[23] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method, Methods. 25 (2001) 402–408.
[24] P. Mukhopadhyay, M. Rajesh, G. Haskó, B.J. Hawkins, M. Madesh, P. Pacher, Simultaneous detection of apoptosis and mitochondrial superoxide production in live cells by flow cytometry and confocal microscopy, Nat. Protoc. 2 (2007) 2295–2301.
[25] B. Morgan, M.C. Sobotta, T.P. Dick, Free Radical Biology & Medicine Measuring E GSH and H2O2 with roGFP2-based redox probes, Free Radic. Biol. Med. 51 (2011) 1943–1951.
[26] D.W. Huang, B.T. Sherman, R.A. Lempicki, Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources, Nat. Protoc. 4 (2009) 44–57.
[27] S.S. Chung, M. Kim, J.S. Lee, B.Y. Ahn, H.S. Jung, H.M. Lee, K.S. Park, C. Ss, M. Kim, L. Js, A. By, J. Hs, L. Hm, P. Ks, Mechanism for antioxidative effects of thiazolidinediones in pancreatic β -cells, Am. J. Physiol. Metab. 301 (2011) 912–921.
[28] H. Yaribeygi, M.T. Mohammadi, A. Sahebkar, D. Res, M.T. Mohammadi, M. Sciences, A. Sahebkar, PPAR-α agonist improves hyperglycemia-induced oxidative stress in pancreatic cells by potentiating antioxidant defense system, Drug Res. (Stuttg). 68 (2018) 355–360.
[29] G.D. Girnun, F.E. Domann, S.A. Moore, M.E.C. Robbins, F. Radical, R.B.P.G.D. G, Identification of a Functional Peroxisome Proliferator-Activated Receptor Response Element in the Rat Catalase Promoter, Mol. Endocrinol. 16 (2002) 2793–2801.
[30] R. Nanbu-wakao, Y. Fujitani, Y. Masuho, M. Muramatu, H. Wakao, Prolactin enhances CCAAT enhancer-binding protein- β (C/ EBP β) and Peroxisome Proliferator-Activated Receptor Gamma (PPAR γ) messenger RNA expression and stimulates adipogenic conversion of NIH-3T3 cells, Mol. Endocrinol. 14 (2000) 307–316.
[31] M.E. Okur, I.D. Karantas, P.I. Siafaka, Diabetes Mellitus : A Review on Pathophysiology , Current Status of Oral Medications and Future Perspectives, Acta Pharm. Sci. 55 (2017) 61–82.
[32] A.T. Kharroubi, H.M. Darwish, Diabetes mellitus : The epidemic of the century, World J. Diabetes. 6 (2015) 850–867.
[33] J. Switala, P.C. Loewen, Diversity of properties among catalases, Arch. Biochem. Biophys. 401 (2002) 145–154.
[34] C. Heit, S. Marshall, S. Singh, X. Yu, G. Charkoftaki, H. Zhao, D.J. Orlicky, K.S. Fritz, D.C. Thompson, A.M. Campus, H. Zhao, Catalase Deletion Promotes PreDiabatic Phenotype in Mice, Free Radic. Biol. Med. 103 (2017) 48–56.
[35] K. Takemoto, M. Tanaka, H. Iwata, R. Nishihara, K. Ishihara, D. Wang, K. Ogino, K. Taniuchi, N. Masuoka, Low catalase activity in blood is associated with the diabetes caused by alloxan, Clin. Chim. Acta. 407 (2009) 43–46.
[36] E. Szaleczky, J. Prechl, J. Fehér, A. Somogyi, Alterations in enzymatic antioxidant defence in diabetes mellitus − a rational approach, Postgrad. Med. J. 75 (1999) 13–17.
[37] E.C. Opara, Oxidative stress , micronutrients , diabetes mellitus and its complications, J. R. Soc. Promot. Health. 122 (2002) 28–34.
[38] G. Wolf, N. Aumann, M. Michalska, A. Bast, J.F. Beck, U. Lendeckel, P. Newsholme, R. Walther, Peroxiredoxin III protects pancreatic b cells from apoptosis, J. Endocrinol. 207 (2010) 163–175.
[39] F.M.M. Paula, S.M. Ferreira, A.C. Boschero, K.L.A. Souza, Molecular and Cellular Endocrinology Modulation of the peroxiredoxin system by cytokines in insulin-producing RINm5F cells: Down-regulation of PRDX6 increases susceptibility of beta cells to oxidative stress, Mol. Cell. Endocrinol. 374 (2013) 56–64.
[40] M.B. Debril, J.P. Renaud, L. Fajas, J. Auwerx, The pleiotropic functions of peroxisome proliferator-activated receptor γ, J. Mol. Med. 79 (2001) 30–47.
[41] H.Y. Yoo, M.S. Chang, H.M. Rho, Induction of the rat Cu / Zn superoxide dismutase gene through the peroxisome proliferator-responsive element by arachidonic acid, Gene. 234

(1999) 87–91.
[42] I. Inoue, S. Goto, T. Matsunaga, T. Nakajima, T. Awata, S. Hokari, T. Komoda, S. Katayama, The ligands/activators for peroxisome proliferator-activated receptor α (PPAR α) and PPAR γ increase Cu 2+ ,Zn 2+ superoxide dismutase and decrease p22phox message expressions in primary endothelial cells, Metabolism. 50 (2001) 3–11.
[43] B. Desvergne, W. Wahli, Peroxisome Proliferator-Activated Receptors : Nuclear Control of Metabolism, Endocr. Rev. 20 (1999) 649–688.
[44] H. Keller, C. Dreyer, J. Medin, M. Abderrahim, K. OZATO, W. Wahli, Fatty acids and retinoids control lipid metabolism through activation of peroxisome proliferator-activated receptor-retinoid X receptor heterodimers, Proc. Natl. Acad. Sci. 90 (1993) 2160–2164.
[45] D. Oropeza, N. Jouvet, K. Bouyakdan, G. Perron, L. Ringuette, L.H. Philipson, R.S. Kiss, V. Poitout, T. Alquier, J.L. Estall, PGC-1 coactivators in b -cells regulate lipid metabolism and are essential for insulin secretion coupled to fatty acids, Mol. Metab. 4 (2015) 811–822.
[46] K. Ravnskjaer, M. Boergesen, B. Rubi, J.K. Larsen, T. Nielsen, J. Fridriksson, P. Maechler, S. Mandrup, Peroxisome Proliferator-Activated Receptor Alpha (PPAR Alpha) Potentiates, whereas PPAR Gamma Attenuates, Glucose- Stimulated Insulin Secretion in Pancreatic Beta-Cells, Endocrinology. 146 (2005) 3266–3276.
[47] T.C. Brelje, N. V. Bhagroo, L.E. Stout, R.L. Sorenson, Beneficial effects of lipids and prolactin on insulin secretion and beta cell proliferation: A role for lipids in the adaptation of islets to pregnancy, J. Endocrinol. 197 (2008) 265–276.
[48] G.C. Yaney, B.E. Corkey, Fatty acid metabolism and insulin secretion in pancreatic beta cells, Diabetologia. 46 (2003) 1297–1312.
[49] J. Cen, E. Sargsyan, P. Bergsten, Fatty acids stimulate insulin secretion from human pancreatic islets at fasting glucose concentrations via mitochondria-dependent and -independent mechanisms, Nutr. Metab. (Lond). 13 (2016) 1–9.
[50] M.G. Rosca, E.J. Vazquez, Q. Chen, J. Kerner, T.S. Kern, C.L. Hoppel, Oxidation of Fatty Acids Is the Source of Increased Mitochondrial Reactive Oxygen Species Production in Kidney Cortical Tubules in Early Diabetes, Diabetes. 61 (2012) 2074–2083.
[51] M. Decker, H. Hofflich, A.N. Elias, Thiazolidinediones and the preservation of beta-cell function, cellular proliferation and apoptosis, Diabetes, Obes. Metab. 10 (2008) 617–625.
[52] D. Gupta, T. Kono3, C. Evans-Molina, The role of peroxisome proliferator-activated receptor γ in pancreatic β cell function and survival: therapeutic implications for the treatment of type 2 diabetes mellitus, Diabetes, Obes. Metab. 12 (2013) 1036–1047.
[53] Y. Kanda, M. Shimoda, S. Hamamoto, K. Tawaramoto, F. Kawasaki, Molecular mechanism by which pioglitazone preserves pancreatic β -cells in obese diabetic mice: evidence for acute and chronic actions as a PPAR γ agonist, Am. J. Physiol. - Endocrinol. Metab. 298 (2010) 278–286.
[54] E. Zender, K. Maedler, D. Bosco, T. Berney, M.Y. Donath, P.A. Halban, Pioglitazone and sodium salicylate protect human β cells against apoptosis and impaired function induced by glucose and interleukin-1 β, J. Clin. Endocrinol. Metab. 89 (2004) 5059–5066.
[55] M. Higa, Y.T. Zhou, M. Ravazzola, D. Baetens, L. Orci, R.H. Unger, Troglitazone prevents mitochondrial alterations, β cell destruction , and diabetes in obese prediabetic rats, Proc. Natl. Acad. Sci. 96 (1999) 11513–11518.
[56] Y. Saitoh, K. Noma, H. Ueno, M. Mizuta, M. Nakazato, Pioglitazone attenuates fatty acid – induced oxidative stress and apoptosis in pancreatic β-cells, Diabetes, Obes. Metab. 10 (2008) 564–573.
[57] L. Li, T. Li, Y. Zhang, Z. Pan, B. Wu, X. Huang, Y. Zhang, Y. Mei, L. Ge, G. Shen, R. Ge, D. Zhu, Y. Lou, Peroxisome proliferator-activated receptor β/δ activation is essential for modulating p-Foxo1/Foxo1 status in functional insulin-positive cell differentiation, Cell Death Dis. 6 (2015) 1–11.
[58] Y. Yagishita, T. Fukutomi, A. Sugawara, H. Kawamura, T. Takahashi, J. Pi, A. Uruno, M. Yamamoto, Nrf2 Protects Pancreatic b -Cells From Oxidative and Nitrosative Stress in Diabetic Model Mice, Diabetes. 63 (2014) 605–618.
[59] J. Pi, Q. Zhangb, J. Fua, C.G. Woods, Y. Hou, B.E. Corkey, S. Collins, M.E. Andersen, ROS signaling, oxidative stress and Nrf2 in pancreatic beta-cell function, Toxicol. Appl.

Pharmacol. 244 (2010) 77–83.
[60] H. Shen, Z. Liu, JNK signaling pathway is a key modulator in cell death mediated by reactive oxygen and nitrogen species, Free Radic. Biol. Med. 40 (2006) 928–939.
[61] H. Kamata, S. Honda, S. Maeda, L. Chang, H. Hirata, M. Karin, Reactive Oxygen Species Promote TNF alpha -Induced Death and Sustained JNK Activation by Inhibiting MAP Kinase Phosphatases, Cell. 120 (2005) 649–661.
[62] F. Brozzi, T.R. Nardelli, M. Lopes, I. Millard, J. Barthson, M. Igoillo-esteve, F.A. Grieco, O. Villate, J.M. Oliveira, M. Casimir, M. Bugliani, F. Engin, G.S. Hotamisligil, P. Marchetti, D.L. Eizirik, Cytokines induce endoplasmic reticulum stress in human , rat and mouse beta cells via different mechanisms, Diabetologia. 58 (2015) 2307–2316.
[63] A. Ammendrup, A. Maillard, K. Nielsen, N.A. Andersen, P. Serup, O.D. Madsen, T. Mandrup-poulsen, C. Bonny, Preferentially Activated by Interleukin-1 and Controls Apoptosis in Differentiating Pancreatic Beta-Cells, Diabetes. 49 (2000) 1468–1476.

4. CONSIDERAÇÕES FINAIS

O estresse oxidativo está entre os fatores que causam danos às células beta pancreáticas e que, consequentemente, levam ao desenvolvimento do DM. A PRL melhora a sobrevivência de células beta frente ao estresse oxidativo, entretanto é desconhecido o mecanismo pelo qual isso ocorre. Uma vez que a PRL modula a expressão gênica de enzimas antioxidantes em diferentes tipos celulares, nosso objetivo foi investigar se a PRL protege as células beta pancreáticas do estresse oxidativo por aumentar a produção de enzimas antioxidantes e qual via de sinalização estaria envolvida nesse efeito.

Demonstramos, pela primeira vez, que a PRL aumenta o conteúdo proteico de enzimas antioxidantes e é capaz de ativar o PPAR em células INS-1E. Entretanto, a PRL não é dependente do PPARγ para proteger as células INS-1E da morte induzida pelo H_2O_2. Sendo assim, pesquisas futuras são necessárias para revelar o exato mecanismo de proteção das células beta pela PRL contra o estresse oxidativo.

5. REFERÊNCIAS GERAIS

ADACHI, T. et al. Effects of PPARgamma ligands and C/EBPbeta enhancer on expression of extracellular-superoxide dismutase. **Redox report : communications in free radical research**, v. 9, n. 4, p. 207–212, 2004. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/15479564.

AMARAL, M. E. C. et al. Prolactin-signal transduction in neonatal rat pancreatic islets and interaction with the insulin-signaling pathway. **Hormone and Metabolic Research**, v. 35, n. 5, p. 282–289, 2003. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/12915997.

AMARAL, M. E. C. et al. Participation of prolactin receptors and phosphatidylinositol 3-kinase and MAP kinase pathways in the increase in pancreatic islet mass and sensitivity to glucose during pregnancy. **Journal of Endocrinology**, v. 183, n. 3, p. 469–476, 2004. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/15590973.

AMERICAN DIABETES ASSOCIATION (ADA). Standard of medical care in diabetes - 2017. **Diabetes Care**, v. 40, p. 4–128, 2017. Disponível em http://care.diabetesjournals.org/content/diacare/suppl/2016/12/15/40.Supplement_1.DC1/DC_40_S1_final.pdf.

ASHCROFT, F. M.; RORSMAN, P. Electrophysiology of the Pancreatic Beta Cell. **Progress in Biophysics & Molecular Biology**, v. 54, p. 57, 1989. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/2484976.

BABIOR, B. M. NADPH oxidase. **Current Opinion in Immunology**, v. 16, n. 1, p. 42–47, 2004. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/14734109.

BALABAN, R. S.; NEMOTO, S.; FINKEL, T. Mitochondria, oxidants, and aging. **Cell**, v. 120, n. 4, p. 483–495, 2005. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/15734681.

BENSELLAM, M. et al. Inhibitor of differentiation proteins protect against oxidative stress by regulating the antioxidant – mitochondrial response in mouse beta cells. **Diabetologia**, v. 58, p. 758–770, 2015. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/25636209.

BERGER, J.; MOLLER, D. E. The mechanisms of action of PPARs. **Annual Review of Medicine**, v. 53, p. 409–435, 2002. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/11818483 .

BERGER, J. P.; AKIYAMA, T. E.; MEINKE, P. T. PPARs: therapeutic targets for metabolic disease. **Trends in Pharmacological Sciences**, v. 26, n. 5, p. 244–251, 2005. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/15860371.

BIENERT, G. P.; SCHJOERRING, J. K.; JAHN, T. P. Membrane transport of hydrogen peroxide. **Biochimica et Biophysica Acta**, v. 1758, p. 994–1003, 2006. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/16566894.

BORDIN, S. et al. Prolactin-modulated gene expression profiles in pancreatic islets from adult female rats. **Molecular and Cellular Endocrinology**, v. 220, n. 1–2, p. 41–50, 2004. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/15196698.

BOSCHERO, A. C. et al. Prolactin Induces Maturation of Glucose Sensing Mechanisms in Cultured Neonatal Rat Islets. **Endocrinology**, v. 133, n. 2, p. 515–520, 1992. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/8344197.

BOSCHERO, A. C. et al. Oxotremorine-m potentiation of glucose-induced insulin release from rat islets involves M3 muscarinic receptors. **The American journal of physiology**, v. 268, n. 2 Pt 1, p. E336-42, 1 fev. 1995. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/7864111.

BRELJE, T. C. et al. Effect of homologous placental lactogens, prolactins, and growth hormones on islet β-cell division and insulin secretion in rat, mouse, and human islets: implication for placental lactogen regulation of islet function during pregnancy. **Endocrinology**, v. 132, n. 2, p. 879–887, 1993. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/8425500.

BRERETON, M. F. et al. Reversible changes in pancreatic islet structure and function produced by elevated blood glucose. **Nature Communications**, v. 5, p. 1–11, 2014. Disponível em https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4143961/.

BRISSOVA, M. et al. Assessment of Human Pancreatic Islet Architecture and Composition by Laser Scanning Confocal Microscopy The Journal of Histochemistry & Cytochemistry. **Journal of histochemistry & cytochemistry**, v. 53, n. 9, p. 1087–1097, 2005. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/15923354.

BROWNLEE, M. Biochemicstry and molecular cell biology of diabetic complications. **Nature**, v. 414, p. 813–820, 2001. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/11742414.

BROWNLEE, M. The pathobiology of diabetic complications: A unifying mechanism. **Diabetes**, v. 54, n. 6, p. 1615–1625, 2005. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/15919781.

CANTLEY, J.; ASHCROFT, F. M. Q&A: Insulin secretion and type 2 diabetes: Why do β-cells fail? **BMC Biology**, v. 13, n. 1, p. 1–7, 2015. Disponível em https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4435650/.

CARVALHEIRA, J. B. C.; ZECCHIN, H. G.; SAAD, M. J. A. Vias de Sinalização da Insulina. **Arquivos Brasileiros de Endocrionologia e Metabologia**, v. 46, n. 4, p. 419–425, 2002. Disponível em http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0004-27302002000400013.

CECARINI, V. et al. Protein oxidation and cellular homeostasis: Emphasis on metabolism. **Biochimica et Biophysica Acta - Molecular Cell Research**, v. 1773, n. 2, p. 93–104, 2007. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/17023064.

CERIELLO, A. Oxidative stress and glycemic regulation. Metabolism, v. 49, n. 2, p. 27-29, 2000. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/10693917.

CHUNG, S. S. et al. Mechanism for antioxidative effects of thiazolidinediones in pancreatic β -cells. **American Journal of Physiology-Endocrinology and Metabolism**, v. 301, p. 912–921, 2011. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/18155663.

CREPALDI, S. C.; CARNEIRO, E. M.; BOSCHERO, A. C. Long-term effect of prolactin treatment on glucose-induced insulin secretion in cultured neonatal rat islets. **Hormone and Metabolic Research**, v. 29, n. 9, p. 220–224, 1997. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/9228206.

DANEMAN, D. Type 1 diabetes. **The Lancet**, v. 367, p. 847–858, 2006. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/16530579.

DE MAZANCOURT, P. et al. Prolactin treatment increases GLUT2 but not the G protein subunit content in cell membranes from cultured neonatal rat islets. **FEBS Letters**, v. 343, n. 2, p. 137–140, 1994. Disponível em

https://www.ncbi.nlm.nih.gov/pubmed/8168618.

DEBRIL, M. B. et al. The pleiotropic functions of peroxisome proliferator-activated receptor γ. **Journal of Molecular Medicine**, v. 79, p. 30–47, 2001. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/11327101.

DODSON, G; STEINER, D. The role of assembly in insulin's biosynthesis. Current Opinion in Structural Biology, v. 8, n. 2, p. 189-94, 1998. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/9631292.

DOLENSEK, J.; RUPNIK, M. S.; STOZER, A. Structural similarities and differences between the human and the mouse pancreas. **Islets**, v. 7, n. 1, p. 2–9, 2015. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/26030186.

EL-BENNA, J. et al. Phagocyte NADPH oxidase: a multicomponent enzyme essential for host defenses. **Archivum immunologiae et therapiae experimentalis**, v. 53, n. 3, p. 199–206, 2005. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/15995580.

ELIASCHEWITZ, F. G. et al. Transplante de ilhotas na prática clínica: estado atual e perspectivas. **Arquivos Brasileiros de Endocrinologia & Metabologia**, v. 53, n. 1, p. 15–23, 2009. Disponível em http://www.scielo.br/pdf/abem/v53n1/v53n1a04.pdf.

EVANS, J. L.; MADDUX, B. A.; GOLDFINE, I. . D. The molecular basis for oxidative stress-induced insulin. **ANTIOXIDANTS & REDOX SIGNALING**, v. 7, p. 1040–1052, 2005. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/15998259.

FERNÁNDEZ-MILLÁN, E. et al. Microbial phenolic metabolites improve glucose-stimulated insulin secretion and protect pancreatic beta cells against tert-butyl hydroperoxide-induced toxicity via ERKs and PKC pathways. **Food and Chemical Toxicology journal**, v. 66, p. 245–253, 2014. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/24491264.

FIORY, F. et al. PED / PEA-15 Inhibits Hydrogen Peroxide- Induced Apoptosis in Ins-1E Pancreatic. **PLoS ONE**, v. 9, n. 12, p. 1–20, 2014. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/25489735.

FOLLI, F. et al. Insulin stimulation of phosphatidylinositol 3-kinase activity and association with insulin receptor substrate 1 in liver and muscle of the intact rat. **The Journal of biological chemistry**, v. 267, n. 31, p. 22171–7, 5 nov. 1992.

Disponível em https://www.ncbi.nlm.nih.gov/pubmed/1385396.

FREEMAN, M. E. et al. Prolactin: structure, function, and regulation of secretion. **Physiological reviews**, v. 80, n. 4, p. 1523-631, 2000. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/11015620.

FREEMARK, M. et al. Targeted deletion of the PRL receptor: Effects on islet development, insulin production, and glucose tolerance. **Endocrinology**, v. 143, n. 4, p. 1378–1385, 2002. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/11897695.

FRIESEN, H.; GUYDA, H.; HARDY, J. Biosynthesis of human growth hormone and prolactin. **The Journal of Clinical Endocrinology & Metabolism**, v. 31, n. 6, p. 611–624, 1970. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/5483096.

FROUD, T. et al. Islet transplantation in type 1 diabetes mellitus using cultured islets and steroid-free immunosuppression: Miami experience. **American Journal of Transplantation**, v. 5, n. 8, p. 2037–2046, 2005. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/15996257.

GIRNUN, G. D. et al. Identification of a Functional Peroxisome Proliferator-Activated Receptor Response Element in the Rat Catalase Promoter. **Molecular Endocrinology**, v. 16, p. 2793–2801, 2002. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/12456800.

GIROTTI, A. W. Lipid hydroperoxide generation, turnover, and effector action in biological systems. **Journal of Lipid Research**, v. 39, p. 1529–1542, 1998. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/9717713.

GOUVEIA, S. et al. Prolactina e metabolismo – uma perspetiva diferente de uma hormona multifuncional. **Revista Portuguesa de Endocrinologia, Diabetes e Metabolismo**, v. 7, n. 2, p. 28–35, 2014. Disponível em https://www.sciencedirect.com/science/article/pii/S1646343916000195.

GROSS, J. L. et al. Diabetes Melito: Diagnóstico, Classificação e Avaliação do Controle Glicêmico. **Arquivos Brasileiros de Endocrinologia & Metabologia**, v. 46, n. 1, p. 16–26, 2002. Disponível em http://www.scielo.br/scielo.php?pid=S0004-27302002000100004&script=sci_abstract&tlng=pt.

GUELHO, D. et al. Prolactina e metabolismo – uma perspetiva diferente de uma hormona multifuncional. **Revista Portuguesa de Endocrinologia, Diabetes e**

Metabolismo, v. 11, n. 2, p. 268–276, 2016. Disponível em https://www.sciencedirect.com/science/article/pii/S1646343916000195.

HABER, E. P. et al. Secreção da Insulina: Efeito Autócrino da Insulina e Modulação por Ácidos Graxos. **Arquivos Brasileiros de Endocrinologia & Metabologia**, v. 45, n. 3, p. 219–227, 2001. Disponível em http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0004-27302001000300003.

HALLIWELL, B. et al. Free Radicals and Antioxidants in Food and In Vivo: What They Do and How They Work. **Critical Reviews in Food Science and Nutrition**, v. 35, n. 1–2, p. 7–20, 1995. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/7748482.

HALLIWELL, B.; GUTTERIDGET, J. M. C. Oxygen toxicity, oxygen radicals, transition metals and disease. **Biochemical Journal**, v. 219, p. 1–14, 1984. Disponível em https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1153442/.

HARDIE, D. G. Organismal Carbohydrate and Lipid Homeostasis. **Cold Spring Harbor Perspectives in Biology**, v. 4, p. 1–16, 2012. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/22550228.

HASKINS, K. et al. Oxidative stress in type 1 diabetes. **Annals new york academy of sciences**, v. 5, p. 43–54, 2003. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/14679039.

HENQUIN, J. Triggering and Amplifying Pathways of Regulation of Insulin Secretion by Glucose. **Diabetes**, v. 49, p. 1751–1760, 2000. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/11078440.

HIGA, M. et al. Troglitazone prevents mitochondrial alterations, β cell destruction , and diabetes in obese prediabetic rats. **Proceedings of the National Academy of Sciences**, v. 96, p. 11513–11518, 1999. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/10500208.

HILFIKER-KLEINER, D. et al. A Cathepsin D-Cleaved 16 kDa Form of Prolactin Mediates Postpartum Cardiomyopathy. **Cell**, v. 128, n. 3, p. 589–600, 2007. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/17289576.

HOTAMISLIGIL, G. S. et al. IRS-1-mediated inhibition of insulin receptor tyrosine kinase activity in TNF-alpha- and obesity-induced insulin resistance. **Science**, v. 271, p. 665–668, 1996. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/8571133.

HUANG, D. W.; SHERMAN, B. T.; LEMPICKI, R. A. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. **Nature Protocols**, v. 4, n. 1, p. 44–57, 2009. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/19131956.

HULSE, R. E.; RALAT, L. A; WEI-JEN, T. Structure, function, and regulation of insulin-degrading enzyme. **Vitamins and hormones**, v. 80, n. 8, p. 635–648, 2009. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/19251053.

HUNT, J. V; DEAN, R. T.; WOLFF, S. P. Hydroxyl radical production and autoxidative glycosylation. Glucose autoxidation as the cause of protein damage in the experimental glycation model of diabetes mellitus and ageing. **The Biochemical journal**, v. 256, n. 1, p. 205–212, 1988. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/2851978.

IBARRA, F. et al. Prolactin, a natriuretic hormone, interacting with the renal dopamine system. **Kidney International**, v. 68, n. 4, p. 1700–1707, 2005. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/16164646.

IDF. International Diabetes Federation. **Diabetes Atlas.** 8th edition. 2015. Disponível em https://www.idf.org/e-library/epidemiology-research/diabetes-atlas.html.

IGNACAK, A. et al. Prolactin - Not only lactotrophin a “new” view of the “old” hormone. **Journal of Physiology and Pharmacology**, v. 63, n. 5, p. 435–443, 2012. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/23211297.

ITOH, K. et al. Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. **Genes & development**, v. 13, p. 76–86, 1999. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/9887101.

JANJIC, D. et al. Free Radical Modulation of Insulin Release in INS-1 Cells Exposed to Alloxan. **Biochemical Pharmacology**, v. 57, p. 639–648, 1999. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/10037448.

JOHANSSON, M. et al. Prolactin treatment improves engraftment and function of transplanted pancreatic islets. **Endocrinology**, v. 150, n. 4, p. 1646–1653, 2009. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/19095742.

KANDA, Y. et al. Molecular mechanism by which pioglitazone preserves pancreatic β -cells in obese diabetic mice: evidence for acute and chronic actions as a PPAR γ agonist. **American Journal of Physiology - Endocrinology and Metabolism**, v. 298, p. 278–286, 2010. Disponível em https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2822485/.

KENSLER, T. W.; WAKABAYASHI, N.; BISWAL, S. Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. **Annual Review of Pharmacology and Toxicology**, v. 47, p. 89–116, 2007. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/16968214.

KORSHUNOV, S. S.; SKULACHEV, V. P.; STARKOV, A. A. High protonic potential actuates a mechanism of production of reactive oxygen species in mitochondria. **FEBS Letters**, v. 416, n. 1, p. 15–18, 1997. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/9369223.

LAIOS, K. et al. Aretaeus of Cappadocia and the first description of diabetes. **Hormones**, v. 11, n. 1, p. 109–113, 2012. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/22450352.

LAKHTAKIA, R. The history of diabetes mellitus. **Medical History**, v. 13, n. August, p. 368–370, 2010. Disponível em https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3749019/.

LEE, B. W. A. N. et al. Dose-related cytoprotective effect of a -lipoic acid on hydrogen peroxide-induced oxidative stress to pancreatic beta cells. **Free Radical Research**, v. 43, n. 1, p. 68–77, 2009. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/19358002.

LEE, J.; KOO, N.; MIN, D. B. Reactive oxygen species, aging, and antioxidative nutraceuticals. **Comprehensive Reviews in Food Science and Food Safety**, v. 3, n. 1, p. 21–33, 2004. Disponível em https://onlinelibrary.wiley.com/doi/abs/10.1111/j.1541-4337.2004.tb00058.x.

LENZEN, S.; DRINKGERN, J.; TIEDGE, M. Low antioxidant enzyme gene expression in pancreatic islets compared with various other mouse tissues. **Free Radical Biology & Medicin**, v. 20, n. 3, p. 463–466, 1996. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/8720919.

LI, J.-M. ROS Generation by Nonphagocytic NADPH Oxidase: Potential Relevance in Diabetic Nephropathy. **Journal of the American Society of Nephrology**, v. 14, p. 221–226, 2003. Disponível em

https://www.ncbi.nlm.nih.gov/pubmed/12874435.

LICHTENBERG, D.; PINCHUK, I. Oxidative stress, the term and the concept. **Biochemical and Biophysical Research Communications**, v. 23, n. April, p. 1–4, 2015. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/25911322.

LIU, L. et al. Uncoupling protein-2 mediates the protective action of berberine against oxidative stress in rat insulinoma INS-1E cells and. **British Journal of Pharmacology**, v. 171, p. 3246–3254, 2014. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/24588674.

LIVAK, K; SCHMITTEGEN, T. D. Analysis of relative rene expression data using real- time quantitative PCR and the $2^{-\Delta\Delta C}$T method. **Methods,** v. 25, p. 402-408, 2001. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/11846609.

LUCA, C. DE; OLEFSKY, J. M. Inflammation and Insulin Resistance. **FEBS Letters**, v. 582, n. 1, p. 97–105, 2008. Disponível em https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1483173/.

LYONS, D. J.; BROBERGER, C. TIDAL WAVES: Network mechanisms in the neuroendocrine control of prolactin release. **Frontiers in Neuroendocrinology**, v. 35, n. 4, p. 420–438, 2014. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/24561279.

MACCRACKEN, J.; HOEL, D.; JOVANOVIS, L. From ants to analogues. Puzzles and promises in diabetes management. **Postgrad Medicine**, v. 101, n. 5, p. 138–140, 1997. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/9126208.

MAECHLER, P.; JORNOT, L.; WOLLHEIM, C. B. Hydrogen Peroxide Alters Mitochondrial Activation and Insulin Secretion in Pancreatic Beta Cells. **The Journal of biological chemistry**, v. 274, p. 27905–27913, 1999. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/10488138.

MARITIM, A. C.; SANDERS, R. A.; WATKINS, J. B. Diabetes, oxidative stress, and antioxidants: A review. **Journal of Biochemical and Molecular Toxicology**, v. 17, n. 1, p. 24–38, 2003. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/12616644.

MARKLUND, S. L. Human copper-containing superoxide dismutase of high molecular weight. **Biochemistry**, v. 79, n. December, p. 7634–7638, 1982. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/6961438.

MARTYN, J. A. J.; KANEKI, M.; YASUHARA, S. Obesity-induced insulin resistence and hyperglycemia: etiological factors and molecular mechanisms. **Anesthesiology**, v. 109, n. 1, p. 137–148, 2008. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/18580184.

MATOUGH, F. A. et al. The Role of Oxidative Stress and Antioxidants in Diabetic Complications. **Medical Journal**, v. 12, n. 1, p. 5–18, 2012. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/22375253.

MCKENNA, N. J.; MALLEY, B. W. O. Combinatorial control of gene expression by nuclear receptors and coregulators. **Cell**, v. 108, p. 465–474, 2002. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/11909518.

MEHMETI, I. et al. ER- resident antioxidative GPx7 and GPx8 enzyme isoforms protect insulin-secreting INS-1E β-cells against lipotoxicity by improving the ER antioxidative capacity. **Free Radical Biology and Medicine**, v. 112, p. 121–130, 2017. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/28751022.

MEHRING, V. J.; MINKOWSKI O. Diabetes mellitus nach Pankreasexstirpation. **Archiv for Experimentelle Pathologie und Pharmakologie**, v. 26, p. 371–387, 1890. Disponível em https://archive.org/details/b28119034.

MELÉNDEZ GARCÍA, R. et al. Prolactin protects retinal pigment epithelium by inhibiting sirtuin 2-dependent cell death. **EBioMedicine**, v. 7, p. 35–49, 2016. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/27322457.

MÖDERSCHEIM, T. A. E. et al. Prolactin is involved in glial responses following a focal injury to the juvenile rat brain. **Neuroscience**, v. 145, n. 3, p. 963–973, 2007. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/17317019.

MONFARED, S. S. M. S.; LARIJANI, B.; ABDOLLAHI, M. Islet transplantation and antioxidant management: a comprehensive review. **World Journal of Gastroenterology**, v. 15, n. 10, p. 1153–1161, 2009. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/19291814.

MORGAN, D. et al. Glucose, palmitate and pro-inflammatory cytokines modulate production and activity of a phagocyte-like NADPH oxidase in rat pancreatic islets and a clonal beta cell line. **Diabetologia**, v. 50, n. 2, p. 359–369, 2007. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/17151863.

MUKHOPADHYAY, P. et al. Simultaneous detection of apoptosis and mitochondrial superoxide production in live cells by flow cytometry and confocal microscopy. **Nature Protocols**, v. 2, n. 9, p. 2295–2301, 2007. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/17853886.

NANBU-WAKAO, R. et al. Prolactin enhances CCAAT enhancer-binding protein- β (C/ EBP β) and Peroxisome Proliferator-Activated Receptor β (PPAR β) messenger RNA expression and stimulates adipogenic conversion of NIH-3T3 cells. **Molecular Endocrinology**, v. 14, n. 2, p. 307–316, 2000. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/10674402.

NARDELLI, T. R. et al. Prolactin protects against cytokine-induced beta cell death by NFkB and JNK inhibition. **Journal of Molecular Endocrinology**, v. 61, n. 1, p. 25–36, 2018. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/29632026.

NATHAN, C.; BUSSEL, A. C. Beyond oxidative stress: an immunologist's guide to reactive oxygen species. **National intitutes of health**, v. 193, n. 1, p. 118–125, 2011. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/23618831.

NEWSHOLME, P. et al. Diabetes associated cell stress and dysfunction: role of mitochondrial and non-mitochondrial ROS production and activity. **The Journal of Physiology**, v. 583, n. 1, p. 9–24, 2007. Disponível em https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2277225/.

ORRENIUS, S.; GOGVADZE, V.; ZHIVOTOVSKY, B. Mitochondrial Oxidative Stress: Implications for Cell Death. **Annual Review of Pharmacology and Toxicology**, v. 47, n. 1, p. 143–183, 2007. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/17029566.

PAEZ-ESPINOSA, E. V. Insulin-induced tyrosine phosphorylation of Shc in liver, muscle and adipose tissue of insulin resistant rats. **Molecular and ellular Endocrinology**, v. 156, p. 121-129,1999. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/10612430.

PAPP, L. V. et al. From selenium to selenoproteins: synthesis, identity, and their role in human health. **Antioxidants & Redox signaling**, v. 9, n. 7, p. 776–806, 2007. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/17508906.

PATTI, M. E.; KAHN, C. R. The insulin receptor - a critical link in glucose homeostasis and insulin action. **Journal of basic and clinical physiology and pharmacology**, v. 9, n. 2–4, p. 89–109, 1998. Disponível em

https://www.ncbi.nlm.nih.gov/pubmed/10212828.

PI, J. et al. Reactive Oxygen Species as a Signal in Glucose- Stimulated Insulin Secretion. **Diabetes**, v. 56, p. 1783–1791, 2007. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/17400930.

POITOUT, V.; ROBERTSON, R. P. Glucolipotoxicity: Fuel excess and β-cell dysfunction. **Endocrine Reviews**, v. 29, n. 3, p. 351–366, 2008. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/18048763.

REPA, J. J.; EVANS, R. M.; MANGELSDORFZI, D. J. Nuclear receptors and lipid physiology: opening the X-Files. **Science**, v. 294, p. 1866–1870, 2001. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/11729302.

RETH, M. Hydrogen peroxide as second messenger in lymphocyte activation. **Nature immunology**, v. 3, n. 12, p. 1129–1134, 2002. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/12447370.

RORSMAN, P. et al. The Cell Physiology of Biphasic Insulin Secretion. **News in physiological sciences**, v. 15, p. 72–77, 2000. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/11390882.

RUTTER, G. A. et al. Pancreatic β-cell identity, glucose sensing and the control of insulin secretion. **Biochemical Journal**, v. 23, n. 2, p. 203–218, 2015. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/25697093.

RYAN, E. A. et al. Successful Islet Transplantation: Continued insulin reserve provides long-term glycemic control. **Diabetes**, v. 51, p. 2148–2157, 2002. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/12086945.

SAHEBKAR, A. et al. Head-to-head comparison of statins versus fibrates in reducing plasma fibrinogen concentrations: A systematic review and meta-analysis. **Pharmacological Research**, v. 103, p. 236–252, 2016. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/26657419.

SAHEBKAR, A. et al. Comparison of the effects of fibrates versus statins on plasma lipoprotein(a) concentrations: A systematic review and meta-analysis of head-to-head randomized controlled trials. **BMC Medicine**, v. 15, n. 1, p. 1–14, 2017. Disponível em https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5290642/.

SAITOH, Y. et al. Pioglitazone attenuates fatty acid – induced oxidative stress and apoptosis in pancreatic β-cells. **Diabetes, Obesity and Metabolism**, v. 10, p. 564–573, 2008. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/17593232.

SALSALI, A.; NATHAN, M. A review of types 1 and 2 diabetes mellitus and their treatment with insulin. **American Journal of Therapeutics**, v. 13, n. 4, p. 349–361, 2006. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/16858171.

SBD. **Diretrizes Sociedade Brasileira de Diabetes**. 2018. Disponível em https://www.diabetes.org.br/profissionais/images/2017/diretrizes/diretrizes-sbd-2017-2018.pdf.

SEMPLE, R. K.; CHATTERJEE, V. K. K.; RAHILLY, S. O. PPAR γ and human metabolic disease. **The Journal of Clinical Investigation**, v. 116, n. 3, p. 581–589, 2006. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/16511590.

SHAWL, A. I.; PARK, K.; KIM, U. Insulin receptor signaling for the proliferation of pancreatic β-cells: Involvement of Ca2+ second messengers, IP3, NAADP and cADPR. **Islets**, v. 1, n. 3, p. 216–223, 2009. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/21099275.

SHEN, X. et al. The effect of FFAR1 on pioglitazone-mediated attenuation of palmitic acid-induced oxidative stress and apoptosis in βTC6 cells. **Metabolism**, v. 63, n. 3, p. 335–351, 2014. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/24360748.

SIES, H. Role of Metabolic H2O2. **The Journal of biological chemistry**, v. 289, n. 13, p. 8735–8741, 2014. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/24515117.

SIES, H. Oxidative stress : a concept in redox biology and medicine. **Redox Biology**, v. 4, p. 180–183, 2015. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/25588755.

SIES, H. Hydrogen peroxide as a central redox signaling molecule in physiological oxidative stress: Oxidative eustress. **Redox Biology**, v. 11, p. 613–619, 2017. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/28110218,

SIES, H.; JONES, D. Oxidative Stress. **Encyclopedia of Stress**, v. 3, p. 45–48, 2007. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/28441057.

SINGH, P. P. et al. Reactive oxygen species, reactive nitrogen species and antioxidants in etiopathogenesis of diabetes mellitus type-2. **Indian journal of clinical biochemistry : IJCB**, v. 24, n. 4, p. 324–42, 2009. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/23105858.

SKELIN, M.; RUPNIK, M.; CENCIC, A. Pancreatic beta cell lines and their applications in diabetes mellitus research. **ALTEX: Alternatives to Animal Experiments**, v. 27, n. 2, p. 105–113, 2010. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/20686743.

SLAMENOVA, D. et al. Comparison of biological processes induced in HepG2 cells by tert -butyl hydroperoxide (t -BHP) and hydroperoxide (H2O2): the influence of carvacrol. **Mutation Research - Genetic Toxicology and Environmental Mutagenesis**, v. 757, n. 1, p. 15–22, 2013. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/23867853.

SORENSON, R. L.; BRELJE, T. C. Adaptation of Islets of Langerhans to Pregnancy: 3-Cell Growth, Enhanced Insulin Secretion and the Role of Lactogenic Hormones. **Hormone and Metabolic Research**, v. 29, p. 301–307, 1997. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/9230352.

STADTMAN, E. R. Protein oxidation in aging and age-related diseases. **Annals of the New York Academy of Sciences**, v. 928, p. 22–38, 2001. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/11795513.

STEWART, W. C. et al. STAT 5 activators can replace the requirement of FBS in the adipogenesis of 3T3-L1 cells. **Biochemical and Biophysical Research Communications**, v. 324, n. 1, p. 355–359, 2004. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/15465026.

SUN, Y. et al. Chronic palmitate exposure inhibits AMPKalpha and decreases glucose-stimulated insulin secretion from beta-cells: modulation by fenofibrate. **Acta pharmacologica Sinica**, v. 29, n. 4, p. 443–450, 2008. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/18358090.

SYKIOTIS, G. .; BOHMANN, D. Stress-activated cap'n'collar transcription factors in aging and human disease. **Science Signaling**, v. 3, n. 112, p. 1–45, 2010. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/20215646.

TACHIBANA, K. et al. Mutation in Insulin Receptor Attenuates Oxidative Stress and Apoptosis in Pancreatic Beta-Cells Induced by Nutrition Excess : Reduced

Insulin Signaling and. **Hormone and Metabolic Research**, v. 47, p. 176–183, 2015. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/25295420.

TANAKA, Y. et al. Prevention of glucose toxicity in HIT-T15 cells and ZDF rats by an antioxidant, N-acetyl-L-cysteine. **Investigative Ophthalmology and Visual Science**, v. 37, n. 3, p. 10857–10862, 1996. Disponível em http://www.pnas.org/content/96/19/10857.

TENGHOLM, A. Cyclic AMP dynamics in the pancreatic β-cell. **Informa Healthcare**, v. 117, n. 4, p. 355–369, 2012. Disponível em https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3497220/.

TERRA, L. F. et al. Recombinant human prolactin promotes human beta cell survival via inhibition of extrinsic and intrinsic apoptosis pathways. **Diabetologia**, v. 54, n. 6, p. 1388–1397, 2011. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/21394492 .

THÉBAULT, S. Potential mechanisms behind the antioxidant actions of prolactin in the retina. **Experimental Eye Research**, v. 160, p. 56–61, 2017. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/28456446.

THOMAS, H. E. et al. Beta cell apoptosis in diabetes. **Apoptosis**, v. 14, n. 12, p. 1389–1404, 2009. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/19322660.

TIEDGE, M. et al. Relation Between Antioxidant Enzyme Gene Expression and Antioxidative Defense Status of Insulin-Producing Cells. **Diabetes**, v. 46, p. 1733–1742, 1997. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/9356019.

TOMIO, A. et al. Prolactin can modulate CD4+ T-cell response through receptor-mediated alterations in the expression of T-bet. **Immunology and cell biology**, v. 86, n. 7, p. 616–621, 2008. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/18414429.

TYAGI, S. et al. The peroxisome proliferator-activated receptor : a family of nuclear receptors role in various diseases. **Journal of Advanced Pharmaceutical Technology and Research**, v. 2, n. 4, p. 236–240, 2011. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/22247890.

VALKO, M. et al. Free radicals and antioxidants in normal physiological functions and human disease. **The International Journal of Biochemistry & Cell Biology**, v. 39, n. 1, p. 44–84, 2007. Disponível em

https://www.ncbi.nlm.nih.gov/pubmed/16978905.

VANDEWALLE, B. et al. PPAR γ -dependent and -independent effects of Rosiglitazone on lipotoxic human pancreatic islets. **Biochemical and Biophysical Research Communications**, v. 366, p. 1096–1101, 2008. Disponível em https://europepmc.org/abstract/med/18155663.

VEGA-MONROY, M.-L. L. DE LA; FERNANDEZ-MEJIA, C. Beta-Cell Function and Failure in Type 1 Diabetes. **Intech: Open Science Open Minds**, v. 6, p. 111–133, 2012. Disponível em https://www.intechopen.com/books/type-1-diabetes-pathogenesis-genetics-and-immunotherapy/beta-cell-function-and-failure-in-type-1-diabetes.

VILLARD, J. Transcription regulation and human diseases. **Swiss Medical Weekly**, v. 134, p. 571–580, 2004. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/15592948.

WAN, Q.-F. et al. Protein Kinase Activation Increases Insulin Secretion by Sensitizing the Secretory Machinery to Ca $^{2+}$. **The Journal of General Physiology**, v. 124, n. 6, p. 653–662, 2004. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/15572345.

WEIR, G. C.; AGUAYO-MAZZUCATO, C.; BONNER-WEIR, S. β-cell dedifferentiation in diabetes is important, but what is it? **Islets**, v. 5, n. 5, p. 233–237, 2013. Disponível em https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4010577/.

WHO. World Health Organization. **Global report on diabetes**. 2016. Disponível em http://apps.who.int/iris/bitstream/handle/10665/204871/9789241565257_eng.pdf;jsessionid=33076149E9ABB1594903A408A25FDBB9?sequence=1. Acesso em:10 nov. 2018.

WHO. World Health Organization. **Diabetes**. Disponível em: http://www.who.int/diabetes/en/. Acesso em:10 nov. 2018.

WOLLHEIM, C. B.; MAECHLER, P. Beta Cell Mitochondria and Insulin Secretion. **Diabetes**, v. 52, n. 3, p. 37–42, 2002. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/11815456.

YAGIHASHI, S. Advances in pathology of diabetes from pancreatic islets to neuropathy — a tribute to Paul Langerhans. **Pathology Internationa**, v. 65, p. 157–169, 2015. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/25708009.

YAMAMOTO, T. et al. β-cell specific cytoprotection by prolactin on human islets. **Transplantation Proceedings**, v. 40, n. 2, p. 382–386, 2009. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/18374075.

YAMAMOTO, T. et al. Prolactin Supplementation to Culture Medium Improves β-Cell Survival. **Transplantation**, v. 89, n. 11, p. 1328–1335, 2010. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/20357700.

YARIBEYGI, H. et al. PPAR-α agonist improves hyperglycemia-induced oxidative stress in pancreatic cells by potentiating antioxidant defense system. **Drug Research**, v. 68, n. 6, p. 355–360, 2018. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/29433142.

YOO, H. Y.; CHANG, M. S.; RHO, H. M. Induction of the rat Cu / Zn superoxide dismutase gene through the peroxisome proliferator-responsive element by arachidonic acid. **Gene**, v. 234, p. 87–91, 1999. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/10393242.

ZENDER, E. et al. Pioglitazone and sodium salicylate protect human β cells against apoptosis and impaired function induced by glucose and interleukin-1 β. **The Journal of Clinical Endocrinology & Metabolism**, v. 89, p. 5059–5066, 2004. Disponível em https://www.ncbi.nlm.nih.gov/pubmed/15472206.

Biochimica et Biophysica Acta (BBA)

GUIDE FOR AUTHORS
Your paper your way

We now differentiate between the requirements for new and revised submissions. You may choose to submit your manuscript as a single Word or PDF file to be used in the refereeing process. Only when your paper is at the revision stage, will you be requested to put your paper in to a 'correct format' for acceptance and provide the items required for the publication of your article.

BBA General Subjects accepts for submission either original, hypothesis-driven studies or reviews covering subjects in **biochemistry** and **biophysics** that are considered to have general interest for a wide audience. Manuscripts with **interdisciplinary** approaches are especially encouraged. Preferred topics include **medically important** biochemistry/biophysics research and **emerging areas** such as **nanobiology** (nanoparticles, nanotoxicology, nanomedicine), **systems biology** (genomics, proteomics, lipidomics, glycomics, bioinformatics based on experimental approaches), **chemical biology** (chemical compounds, drug mechanisms, synthesis of novel compounds, click chemistry), **structural biology** (crystallography, NMR, multimeric proteins, protein dynamics), **novel complexes** (pure natural compounds, synthetic compounds, protein complexes, nucleic acid derivatives), **cellular signaling** (receptor signaling, protein phosphorylation cascades, phosphatases, secondary messengers, transcription regulation, gene expression), **glycobiology** (sugar metabolites and metabolism, glycosylated proteins, membrane protein, glycosylation, glycomics), **redox biology** (redox switches, glutathione and thioredoxin systems, oxygen and nitrogen radical species, superoxide, hydrogen peroxide, hydroxyl radical, nitric oxide, peroxides, hypoxia, redox regulation of transcription factors), **neurobiology** (neuronal growth factors and nerve signaling, glial cells, autonomic and central nervous systems), **stem cells** (differentiation, stem cell isolation and cultivation, growth factors), **imaging methodologies** and **mechanistic characterization** of compounds having biochemical importance and general interest (drug leads, toxicants, nutrients, metabolites).

Authors must provide a sentence at submission stating the reason that the work described in the manuscript has **general significance and interest** to a **wide audience**. Claims of novelty do not suffice. The author statement will be used as an aid in the reviewing process and will be included in the final version of the accepted paper to orient readers of *BBA General Subjects*.

Structural data
For papers describing structures of biological macromolecules, the atomic coordinates and the related experimental data (structure factor amplitudes/intensities and/or NMR restraints) must be deposited at a member site of the Worldwide Protein Data Bank (http://www.wwpdb.org): RCSB PDB (http://www.pdb.org), MSD-EBI (http://www.ebi.ac.uk/pdbe/ , PDBj (http://www.pdbj.org), or BMRB (http://www.bmrb.wisc.edu). Manuscripts must carry

a statement that coordinates and structure factors (or NMR restraints) have been deposited in the Protein Data Bank. The accession number(s) must be cited in the manuscript at the end of the Materials and Methods section. Authors must agree to release the atomic coordinates and experimental data immediately upon publication.

It is increasingly common for coordinates to be deposited in the Protein Data Bank without an associated publication. Before submission to BBA, authors are expected to search the Protein Data Bank for related structures using one or more alignment programs and report the outcome. Prior deposition of related coordinates, without an associated publication, does not necessarily preclude publication in BBA. The primary criteria for publication of a structure in BBA are that it provides novel structural insights or important new functional and biological insights that are likely to be of general interest.

Types of paper

Reviews and Mini-Reviews are typically commissioned by the Editors. All Review Articles should be authoritative, state-of-the-art accounts of the selected research field, be of high interest, balanced and accurate. Beyond summaries of important scientific developments and ideas, authors are encouraged to identify and discuss how the field may be impacted or develop in the future, including insights that may be of significance to the scientific community. All BBA Review Articles undergo rigorous and full peer review, in the same way as regular research papers, and publication cannot be guaranteed. The number of co-authors of review articles is limited to five and each author is expected to make a substantial contribution to the writing of the manuscript.

Unsolicited reviews will be considered only in exceptional cases and should be preceded by a letter of enquiry from the prospective author, who should be a recognized expert in the field of the proposed article. Pre-submission enquires may be sent to the Editorial Office bbareviews@elsevier.com. Please be sure to specify which one of the ten BBA journals you request to consider your proposal.

Specifically, authors must provide the following in their review proposal: Both your own and any co- author(s) affiliation and full contact details An explanation of the current interest and significance to the broad readership of the journal, that is, compelling reasons why the review should be considered A 500-600 word summary which clearly outlines what will be discussed in the article, plus up to 20 key references that indicate the intended breadth of the proposed article (please note that references should include work published in the past 2-4 years) Only proposals that include this information will be considered.

Contact details for submission

Papers should be submitted using the BBA General Subjects online submission system, http://ees.elsevier.com/bbagen. For questions on the submission and reviewing process, please contact the Editorial Office at bbagen@elsevier.com.

BEFORE YOU BEGIN

Ethics in publishing

Please see our information pages on Ethics in publishing and Ethical guidelines for journal publication.

Studies in humans and animals
If the work involves the use of human subjects, the author should ensure that the work described has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. The manuscript should be in line with the Recommendations for the Conduct, Reporting, Editing and Publication of Scholarly Work in Medical Journals and aim for the inclusion of representative human populations (sex, age and ethnicity) as per those recommendations. The terms sex and gender should be used correctly.

Authors should include a statement in the manuscript that informed consent was obtained for experimentation with human subjects. The privacy rights of human subjects must always be observed.

All animal experiments should comply with the ARRIVE guidelines and should be carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments, or the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) and the authors should clearly indicate in the manuscript that such guidelines have been followed. The sex of animals must be indicated, and where appropriate, the influence (or association) of sex on the results of the study.

Conflict of interest
BBA General Subjects follows the ICMJE recommendations regarding conflict of interest disclosures. All authors are required to report the following information with each submission: (1) All third-party financial support for the work in the submitted manuscript. (2) All financial relationships with any entities that could be viewed as relevant to the general area of the submitted manuscript. (3) All sources of revenue with relevance to the submitted work who made payments to you, or to your institution on your behalf, in the 36 months prior to submission. (4) Any other interactions with the sponsor of outside of the submitted work should also be reported. (5) Any relevant patents or copyrights (planned, pending, or issued). (6) Any other relationships or affiliations that may be perceived by readers to have influenced, or give the appearance of potentially influencing, what you wrote in the submitted work. As a general guideline, it is usually better to disclose a relationship than not. This information will be acknowledged at publication in a Transparency Document link directly in the article. Additional information on the ICMJE recommendations can be found at: http://www.icmje.org/. The form for conflict of interest disclosure can be downloaded here: http://www.icmje.org/coi_disclosure.pdf (if this link does not display properly in your browser, please right-click the link and select "Save Target As..." or "Save Link as..." from the pop-up menu).

Submission declaration and verification
Submission of an article implies that the work described has not been published previously (except in the form of an abstract, a published lecture or academic thesis, see 'Multiple, redundant or concurrent publication' for more information), that it is not under consideration for publication elsewhere, that its publication is approved by all

authors and tacitly or explicitly by the responsible authorities where the work was carried out, and that, if accepted, it will not be published elsewhere in the same form, in English or in any other language, including electronically without the written consent of the copyright- holder. To verify originality, your article may be checked by the originality detection service Crossref Similarity Check.

Preprints
Please note that preprints can be shared anywhere at any time, in line with Elsevier's sharing policy. Sharing your preprints e.g. on a preprint server will not count as prior publication (see 'Multiple, redundant or concurrent publication' for more information).

Use of inclusive language
Inclusive language acknowledges diversity, conveys respect to all people, is sensitive to differences, and promotes equal opportunities. Articles should make no assumptions about the beliefs or commitments of any reader, should contain nothing which might imply that one individual is superior to another on the grounds of race, sex, culture or any other characteristic, and should use inclusive language throughout. Authors should ensure that writing is free from bias, for instance by using 'he or she', 'his/her' instead of 'he' or 'his', and by making use of job titles that are free of stereotyping (e.g. 'chairperson' instead of 'chairman' and 'flight attendant' instead of 'stewardess').

Author contributions
For transparency, we encourage authors to submit an author statement file outlining their individual contributions to the paper using the relevant CRediT roles: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Software; Supervision; Validation; Visualization; Roles/Writing - original draft; Writing - review & editing. Authorship statements should be formatted with the names of authors first and CRediT role(s) following.

Authorship
All authors should have made substantial contributions to all of the following: (1) the conception and design of the study, or acquisition of data, or analysis and interpretation of data, (2) drafting the article or revising it critically for important intellectual content, (3) final approval of the version to be submitted.

Changes to authorship
Authors are expected to consider carefully the list and order of authors **before** submitting their manuscript and provide the definitive list of authors at the time of the original submission. Any addition, deletion or rearrangement of author names in the authorship list should be made only **before** the manuscript has been accepted and only if approved by the journal Editor. To request such a change, the Editor must receive the following from the **corresponding author**: (a) the reason for the change in author list and (b) written confirmation (e-mail, letter) from all authors that they agree with the addition, removal or rearrangement. In the case of addition or removal of authors, this includes confirmation from the author being added or removed.

Only in exceptional circumstances will the Editor consider the addition, deletion or

rearrangement of authors **after** the manuscript has been accepted. While the Editor considers the request, publication of the manuscript will be suspended. If the manuscript has already been published in an online issue, any requests approved by the Editor will result in a corrigendum.

Article transfer service
This journal is part of our Article Transfer Service. This means that if the Editor feels your article is more suitable in one of our other participating journals, then you may be asked to consider transferring the article to one of those. If you agree, your article will be transferred automatically on your behalf with no need to reformat. Please note that your article will be reviewed again by the new journal. More information.

Copyright
Upon acceptance of an article, authors will be asked to complete a 'Journal Publishing Agreement' (see more information on this). An e-mail will be sent to the corresponding author confirming receipt of the manuscript together with a 'Journal Publishing Agreement' form or a link to the online version of this agreement.

Subscribers may reproduce tables of contents or prepare lists of articles including abstracts for internal circulation within their institutions. Permission of the Publisher is required for resale or distribution outside the institution and for all other derivative works, including compilations and translations. If excerpts from other copyrighted works are included, the author(s) must obtain written permission from the copyright owners and credit the source(s) in the article. Elsevier has preprinted forms for use by authors in these cases.

For gold open access articles: Upon acceptance of an article, authors will be asked to complete an 'Exclusive License Agreement' (more information). Permitted third party reuse of gold open access articles is determined by the author's choice of user license.

Author rights
As an author you (or your employer or institution) have certain rights to reuse your work. More information.

Elsevier supports responsible sharing
Find out how you can share your research published in Elsevier journals.

Role of the funding source
You are requested to identify who provided financial support for the conduct of the research and/or preparation of the article and to briefly describe the role of the sponsor(s), if any, in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the article for publication. If the funding source(s) had no such involvement then this should be stated.

Funding body agreements and policies
Elsevier has established a number of agreements with funding bodies which allow authors to comply with their funder's open access policies. Some funding bodies will reimburse the author for the gold open access publication fee. Details of existing

agreements are available online.

Open access
This journal offers authors a choice in publishing their research:

Subscription
- Articles are made available to subscribers as well as developing countries and patient groups through our universal access programs.
- No open access publication fee payable by authors.
- The Author is entitled to post the accepted manuscript in their institution's repository and make this public after an embargo period (known as green Open Access). The published journal article cannot be shared publicly, for example on ResearchGate or Academia.edu, to ensure the sustainability of peer- reviewed research in journal publications. The embargo period for this journal can be found below. ***Gold open access***
- Articles are freely available to both subscribers and the wider public with permitted reuse.
- A gold open access publication fee is payable by authors or on their behalf, e.g. by their research funder or institution.

Regardless of how you choose to publish your article, the journal will apply the same peer review criteria and acceptance standards.

For gold open access articles, permitted third party (re)use is defined by the following Creative Commons user licenses:

Creative Commons Attribution (CC BY)
Lets others distribute and copy the article, create extracts, abstracts, and other revised versions, adaptations or derivative works of or from an article (such as a translation), include in a collective work (such as an anthology), text or data mine the article, even for commercial purposes, as long as they credit the author(s), do not represent the author as endorsing their adaptation of the article, and do not modify the article in such a way as to damage the author's honor or reputation.

Creative Commons Attribution-NonCommercial-NoDerivs (CC BY-NC-ND)
For non-commercial purposes, lets others distribute and copy the article, and to include in a collective work (such as an anthology), as long as they credit the author(s) and provided they do not alter or modify the article.
The gold open access publication fee for this journal is **USD 4050**, excluding taxes.

For all articles submitted before **31-Jan-2019**, there is a **33%** discount off the open access publication fee so authors pay **USD 2700**, excluding taxes. Learn more about Elsevier's pricing policy: https://www.elsevier.com/openaccesspricing.

Green open access
Authors can share their research in a variety of different ways and Elsevier has a number of green open access options available. We recommend authors see our open access page for further information. Authors can also self-archive their manuscripts immediately and enable public access from their institution's repository after an embargo period. This is the version that has been accepted for publication

and which typically includes author-incorporated changes suggested during submission, peer review and in editor-author communications. Embargo period: For subscription articles, an appropriate amount of time is needed for journals to deliver value to subscribing customers before an article becomes freely available to the public. This is the embargo period and it begins from the date the article is formally published online in its final and fully citable form. Find out more.
This journal has an embargo period of 12 months.

Elsevier Researcher Academy
Researcher Academy is a free e-learning platform designed to support early and mid-career researchers throughout their research journey. The "Learn" environment at Researcher Academy offers several interactive modules, webinars, downloadable guides and resources to guide you through the process of writing for research and going through peer review. Feel free to use these free resources to improve your submission and navigate the publication process with ease.

Language (usage and editing services)
Please write your text in good English (American or British usage is accepted, but not a mixture of these). Authors who feel their English language manuscript may require editing to eliminate possible grammatical or spelling errors and to conform to correct scientific English may wish to use the English Language Editing service available from Elsevier's WebShop.

Submission
Our online submission system guides you stepwise through the process of entering your article details and uploading your files. The system converts your article files to a single PDF file used in the peer-review process. Editable files (e.g., Word, LaTeX) are required to typeset your article for final publication. All correspondence, including notification of the Editor's decision and requests for revision, is sent by e-mail.

Referees
During the submission process, you will be required to provide the names, addresses, and e-mail addresses of 4 potential referees, as well as a brief description of their expertise relevant to your manuscript. Suggested reviewers should be individuals qualified to evaluate the work you have submitted. The reviewers suggested may not be current, recent or extensive collaborators of yours, and cannot have been involved in the preparation of the manuscript. Please note that the editor retains the sole right to decide whether or not the suggested reviewers are used.

Authors may request exclusion of certain referees if conflicts of interest are anticipated. However, no more than 3 such names should be given. Entire groups or institutions ***cannot*** *be specified for exclusion.*

PREPARATION
New submissions
Submission to this journal proceeds totally online and you will be guided stepwise through the creation and uploading of your files. The system automatically converts your files to a single PDF file, which is used in the peer-review process.

As part of the Your Paper Your Way service, you may choose to submit your manuscript as a single file to be used in the refereeing process. This can be a PDF file or a Word document, in any format or lay- out that can be used by referees to evaluate your manuscript. It should contain high enough quality figures for refereeing. If you prefer to do so, you may still provide all or some of the source files at the initial submission. Please note that individual figure files larger than 10 MB must be uploaded separately.

References
There are no strict requirements on reference formatting at submission. References can be in any style or format as long as the style is consistent. Where applicable, author(s) name(s), journal title/ book title, chapter title/article title, year of publication, volume number/book chapter and the article number or pagination must be present. Use of DOI is highly encouraged. The reference style used by the journal will be applied to the accepted article by Elsevier at the proof stage. Note that missing data will be highlighted at proof stage for the author to correct.

Formatting requirements
There are no strict formatting requirements but all manuscripts must contain the essential elements needed to convey your manuscript, for example Abstract, Keywords, Introduction, Materials and Methods, Results, Conclusions, Artwork and Tables with Captions.

If your article includes any Videos and/or other Supplementary material, this should be included in your initial submission for peer review purposes.

Divide the article into clearly defined sections. Please ensure your paper includes page numbers - this is an essential peer review requirement.

Figures and tables embedded in text
Please ensure the figures and the tables included in the single file are placed next to the relevant text in the manuscript, rather than at the bottom or the top of the file. The corresponding caption should be placed directly below the figure or table.

Peer review
This journal operates a single blind review process. All contributions will be initially assessed by the editor for suitability for the journal. Papers deemed suitable are then typically sent to a minimum of two independent expert reviewers to assess the scientific quality of the paper. The Editor is responsible for the final decision regarding acceptance or rejection of articles. The Editor's decision is final. More information on types of peer review.

REVISED SUBMISSIONS
Use of word processing software
Regardless of the file format of the original submission, at revision you must provide us with an editable file of the entire article. Keep the layout of the text as simple as possible. Most formatting codes will be removed and replaced on processing the article. The electronic text should be prepared in a way very similar to that of conventional manuscripts (see also the Guide to Publishing with Elsevier). See also

the section on Electronic artwork.

To avoid unnecessary errors you are strongly advised to use the 'spell-check' and 'grammar-check' functions of your word processor.

Article structure

Subdivision - numbered sections

Divide your article into clearly defined and numbered sections. Subsections should be numbered 1.1 (then 1.1.1, 1.1.2, ...), 1.2, etc. (the abstract is not included in section numbering). Use this numbering also for internal cross-referencing: do not just refer to 'the text'. Any subsection may be given a brief heading. Each heading should appear on its own separate line.

Introduction

State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

Material and methods

Provide sufficient details to allow the work to be reproduced by an independent researcher. Methods that are already published should be summarized, and indicated by a reference. If quoting directly from a previously published method, use quotation marks and also cite the source. Any modifications to existing methods should also be described.

Results

Results should be clear and concise.

Discussion

This should explore the significance of the results of the work, not repeat them. A combined Results and Discussion section is often appropriate. Avoid extensive citations and discussion of published literature.

Conclusions

The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of a Discussion or Results and Discussion section.

Appendices

If there is more than one appendix, they should be identified as A, B, etc. Formulae and equations in appendices should be given separate numbering: Eq. (A.1), Eq. (A.2), etc.; in a subsequent appendix, Eq. (B.1) and so on. Similarly for tables and figures: Table A.1; Fig. A.1, etc.

Essential title page information

- ***Title.*** Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.
- ***Author names and affiliations.*** Please clearly indicate the given name(s) and family name(s) of each author and check that all names are accurately spelled. You can add your name between parentheses in your own script behind the English transliteration. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lower- case superscript letter

immediately after the author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name and, if available, the e-mail address of each author.

- ***Corresponding author.*** Clearly indicate who will handle correspondence at all stages of refereeing and publication, also post-publication. This responsibility includes answering any future queries about Methodology and Materials. **Ensure that the e-mail address is given and that contact details are kept up to date by the corresponding author.**
- ***Present/permanent address.*** If an author has moved since the work described in the article was done, or was visiting at the time, a 'Present address' (or 'Permanent address') may be indicated as a footnote to that author's name. The address at which the author actually did the work must be retained as the main, affiliation address. Superscript Arabic numerals are used for such footnotes.

Abstract

Page 2 of the typescript should be reserved for the Abstract which should have no more than 250 words. For Regular Articles, this should follow a structured format, sub-divided into subsections entitled "Background"; "Methods"; "Results"; "Conclusions" and "General Significance". Each subsection should be brief and informative, emphasizing those points that are unique to the paper. Review Articles should also contain a Structured Abstract, sub-divided into subsections entitled "Background"; "Scope of Review"; "Major Conclusions"; and "General Significance". Since summaries are increasingly used by abstracting services which will cut off after a fixed number of words, it is important not to exceed the maximum number of words and to avoid bibliographic references and non-standard abbreviations.

Graphical abstract

Although a graphical abstract is optional, its use is encouraged as it draws more attention to the online article. The graphical abstract should summarize the contents of the article in a concise, pictorial form designed to capture the attention of a wide readership. Graphical abstracts should be submitted as a separate file in the online submission system. Image size: Please provide an image with a minimum of 531 × 1328 pixels (h × w) or proportionally more. The image should be readable at a size of 5 × 13 cm using a regular screen resolution of 96 dpi. Preferred file types: TIFF, EPS, PDF or MS Office files. You can view Example Graphical Abstracts on our information site. Authors can make use of Elsevier's Illustration Services to ensure the best presentation of their images and in accordance with all technical requirements.

Highlights

Highlights are mandatory for this journal. They consist of a short collection of bullet points that convey the core findings of the article and should be submitted in a separate editable file in the online submission system. Please use 'Highlights' in the file name and include 3 to 5 bullet points (maximum 85 characters, including spaces, per bullet point). You can view example Highlights on our information site.

Keywords

Immediately after the abstract, provide a maximum of 6 keywords, using American spelling and avoiding general and plural terms and multiple concepts (avoid, for example, 'and', 'of'). Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible. These keywords will be used for indexing

purposes.

Abbreviations
Define abbreviations that are not standard in this field in a footnote to be placed on the first page of the article. Such abbreviations that are unavoidable in the abstract must be defined at their first mention there, as well as in the footnote. Ensure consistency of abbreviations throughout the article.

Acknowledgements
Collate acknowledgements in a separate section at the end of the article before the references and do not, therefore, include them on the title page, as a footnote to the title or otherwise. List here those individuals who provided help during the research (e.g., providing language help, writing assistance or proof reading the article, etc.).

Formatting of funding sources
List funding sources in this standard way to facilitate compliance to funder's requirements:

Funding: This work was supported by the National Institutes of Health [grant numbers xxxx, yyyy]; the Bill & Melinda Gates Foundation, Seattle, WA [grant number zzzz]; and the United States Institutes of Peace [grant number aaaa].

It is not necessary to include detailed descriptions on the program or type of grants and awards. When funding is from a block grant or other resources available to a university, college, or other research institution, submit the name of the institute or organization that provided the funding.

If no funding has been provided for the research, please include the following sentence:

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Standards for Reporting Enzymology Data (STRENDA)
This journal follows the recommendations of the STRENDA (**St**andards for **R**eporting **En**zymology **Da**ta) Commission of the Beilstein-Institut for the reporting of kinetic and equilibrium binding data. Detailed guidelines can be found at (http://www.strenda.org/documents.html) or in this pdf file.

All reports of kinetic and binding data must include a description of the identity of the catalytic or binding entity (enzyme, protein, nucleic acid or other molecule). This information should include the origin or source of the molecule, its purity, composition, and other characteristics such as post-translational modifications, mutations, and any modifications made to facilitate expression or purification. The assay methods and exact experimental conditions of the assay must be fully described if it is a new assay or provided as a reference to previously published work, with or without modifications. The temperature, pH and pressure (if other than atmospheric) of the assay **must** always be included, even if previously published. In instances where catalytic activity or binding cannot be detected, an estimate of the limit of detection based on the sensitivity and error analysis of the assay should

be provided. Ambiguous terms such as "not detectable" should be avoided. A description of the software used for data analysis should be included along with calculated errors for all parameters.
First-order and second-order rate constants: see pdf for full instructions.

Math formulae
Please submit math equations as editable text and not as images. Present simple formulae in line with normal text where possible and use the solidus (/) instead of a horizontal line for small fractional terms, e.g., *X*/*Y*. In principle, variables are to be presented in italics. Powers of e are often more conveniently denoted by exp. Number consecutively any equations that have to be displayed separately from the text (if referred to explicitly in the text).

Footnotes
Footnotes should be used sparingly. Number them consecutively throughout the article. Many word processors build footnotes into the text, and this feature may be used. Should this not be the case, indicate the position of footnotes in the text and present the footnotes themselves separately at the end of the article.

Artwork
Image manipulation
While it is accepted that authors sometimes need to manipulate images for clarity, manipulation for purposes of deception or fraud will be seen as scientific ethical abuse and will be dealt with accordingly. For graphical images, this journal is applying the following policy: no specific feature within an image may be enhanced, obscured, moved, removed, or introduced. Adjustments of brightness, contrast, or color balance are acceptable as long as they are applied to the entire image and do not obscure or eliminate any information present in the original. Nonlinear adjustments (e.g. changes to gamma settings) must be disclosed in the figure legend.

Digital images in manuscripts nearing acceptance for publication may be scrutinized for any indication of improper manipulation. *BBA General Subjects* reserves the right to ask for original data or images and, if these are not satisfactory, we may decide not to accept the manuscript.

Electronic artwork General points
- Make sure you use uniform lettering and sizing of your original artwork.
- Preferred fonts: Arial (or Helvetica), Times New Roman (or Times), Symbol, Courier.
- Number the illustrations according to their sequence in the text.
- Use a logical naming convention for your artwork files.
- Indicate per figure if it is a single, 1.5 or 2-column fitting image.
- For Word submissions only, you may still provide figures and their captions, and tables within a single file at the revision stage.
- Please note that individual figure files larger than 10 MB must be provided in separate source files. A detailed guide on electronic artwork is available.

Formats
Regardless of the application used, when your electronic artwork is finalized, please 'save as' or convert the images to one of the following formats (note the resolution

requirements for line drawings, halftones, and line/halftone combinations given below): EPS (or PDF): Vector drawings. Embed the font or save the text as 'graphics'. TIFF (or JPG): Color or grayscale photographs (halftones): always use a minimum of 300 dpi. TIFF (or JPG): Bitmapped line drawings: use a minimum of 1000 dpi. TIFF (or JPG): Combinations bitmapped line/half-tone (color or grayscale): a minimum of 500 dpi is required.

Please do not:

- Supply files that are optimized for screen use (e.g., GIF, BMP, PICT, WPG); the resolution is too low.
- Supply files that are too low in resolution.
- Submit graphics that are disproportionately large for the content.

Color artwork

Please make sure that artwork files are in an acceptable format (TIFF (or JPEG), EPS (or PDF) or MS Office files) and with the correct resolution. If, together with your accepted article, you submit usable color figures then Elsevier will ensure, at no additional charge, that these figures will appear in color online (e.g., ScienceDirect and other sites) in addition to color reproduction in print. Further information on the preparation of electronic artwork.

Figure captions

Ensure that each illustration has a caption. A caption should comprise a brief title (**not** on the figure itself) and a description of the illustration. Keep text in the illustrations themselves to a minimum but explain all symbols and abbreviations used.

Tables

Please submit tables as editable text and not as images. Tables can be placed either next to the relevant text in the article, or on separate page(s) at the end. Number tables consecutively in accordance with their appearance in the text and place any table notes below the table body. Be sparing in the use of tables and ensure that the data presented in them do not duplicate results described elsewhere in the article. Please avoid using vertical rules and shading in table cells.

References

Citation in text

Please ensure that every reference cited in the text is also present in the reference list (and vice versa). Any references cited in the abstract must be given in full. Unpublished results and personal communications are not recommended in the reference list, but may be mentioned in the text. If these references are included in the reference list they should follow the standard reference style of the journal and should include a substitution of the publication date with either 'Unpublished results' or 'Personal communication'. Citation of a reference as 'in press' implies that the item has been accepted for publication.

Web references

As a minimum, the full URL should be given and the date when the reference was last accessed. Any further information, if known (DOI, author names, dates, reference to a source publication, etc.), should also be given. Web references can

be listed separately (e.g., after the reference list) under a different heading if desired, or can be included in the reference list.

Data references
This journal encourages you to cite underlying or relevant datasets in your manuscript by citing them in your text and including a data reference in your Reference List. Data references should include the following elements: author name(s), dataset title, data repository, version (where available), year, and global persistent identifier. Add [dataset] immediately before the reference so we can properly identify it as a data reference. The [dataset] identifier will not appear in your published article.

References in a special issue
Please ensure that the words 'this issue' are added to any references in the list (and any citations in the text) to other articles in the same Special Issue.

Reference management software
Most Elsevier journals have their reference template available in many of the most popular reference management software products. These include all products that support Citation Style Language styles, such as Mendeley and Zotero, as well as EndNote. Using the word processor plug-ins from these products, authors only need to select the appropriate journal template when preparing their article, after which citations and bibliographies will be automatically formatted in the journal's style. If no template is yet available for this journal, please follow the format of the sample references and citations as shown in this Guide. If you use reference management software, please ensure that you remove all field codes before submitting the electronic manuscript. More information on how to remove field codes.

Users of Mendeley Desktop can easily install the reference style for this journal by clicking the following link: http://open.mendeley.com/use-citation-style/bba-general-subjects. When preparing your manuscript, you will then be able to select this style using the Mendeley plug- ins for Microsoft Word or LibreOffice.

Reference formatting
There are no strict requirements on reference formatting at submission. References can be in any style or format as long as the style is consistent. Where applicable, author(s) name(s), journal title/ book title, chapter title/article title, year of publication, volume number/book chapter and the article number or pagination must be present. Use of DOI is highly encouraged. The reference style used by the journal will be applied to the accepted article by Elsevier at the proof stage. Note that missing data will be highlighted at proof stage for the author to correct. If you do wish to format the references yourself they should be arranged according to the following examples:

Reference style
Text: Indicate references by number(s) in square brackets in line with the text. The actual authors can be referred to, but the reference number(s) must always be given.
Example: '..... as demonstrated [3,6]. Barnaby and Jones [8] obtained a different result ....'

List: Number the references (numbers in square brackets) in the list in the order in which they appear in the text.
Examples:
Reference to a journal publication:
[1] J. van der Geer, J.A.J. Hanraads, R.A. Lupton, The art of writing a scientific article, J. Sci. Commun. 163 (2010) 51–59. https://doi.org/10.1016/j.Sc.2010.00372.
Reference to a journal publication with an article number:
[2] Van der Geer, J., Hanraads, J.A.J., Lupton, R.A., 2018. The art of writing a scientific article. Heliyon. 19, e00205. https://doi.org/10.1016/j.heliyon.2018.e00205.
Reference to a book:
[3] W. Strunk Jr., E.B. White, The Elements of Style, fourth ed., Longman, New York, 2000. Reference to a chapter in an edited book:
[4] G.R. Mettam, L.B. Adams, How to prepare an electronic version of your article, in: B.S. Jones, R.Z. Smith (Eds.), Introduction to the Electronic Age, E-Publishing Inc., New York, 2009, pp. 281–304. Reference to a website:
[5] Cancer Research UK, Cancer statistics reports for the UK. http://www.cancerresearchuk.org/ aboutcancer/statistics/cancerstatsreport/, 2003 (accessed 13 March 2003).
Reference to a dataset:
[dataset] [6] M. Oguro, S. Imahiro, S. Saito, T. Nakashizuka, Mortality data for Japanese oak wilt disease and surrounding forest compositions, Mendeley Data, v1, 2015. https://doi.org/10.17632/ xwj98nb39r.1.

Journal abbreviations source
Journal names should be abbreviated according to the List of Title Word Abbreviations.

Video
Elsevier accepts video material and animation sequences to support and enhance your scientific research. Authors who have video or animation files that they wish to submit with their article are strongly encouraged to include links to these within the body of the article. This can be done in the same way as a figure or table by referring to the video or animation content and noting in the body text where it should be placed. All submitted files should be properly labeled so that they directly relate to the video file's content. . In order to ensure that your video or animation material is directly usable, please provide the file in one of our recommended file formats with a preferred maximum size of 150 MB per file, 1 GB in total. Video and animation files supplied will be published online in the electronic version of your article in Elsevier Web products, including ScienceDirect. Please supply 'stills' with your files: you can choose any frame from the video or animation or make a separate image. These will be used instead of standard icons and will personalize the link to your video data. For more detailed instructions please visit our video instruction pages. Note: since video and animation cannot be embedded in the print version of the journal, please provide text for both the electronic and the print version for the portions of the article that refer to this content.

Data visualization
Include interactive data visualizations in your publication and let your readers interact and engage more closely with your research. Follow the instructions here to find out about available data visualization options and how to include them with

your article.

Supplementary material
Supplementary material such as applications, images and sound clips, can be published with your article to enhance it. Submitted supplementary items are published exactly as they are received (Excel or PowerPoint files will appear as such online). Please submit your material together with the article and supply a concise, descriptive caption for each supplementary file. If you wish to make changes to supplementary material during any stage of the process, please make sure to provide an updated file. Do not annotate any corrections on a previous version. Please switch off the 'Track Changes' option in Microsoft Office files as these will appear in the published version.

Research data
This journal encourages and enables you to share data that supports your research publication where appropriate, and enables you to interlink the data with your published articles. Research data refers to the results of observations or experimentation that validate research findings. To facilitate reproducibility and data reuse, this journal also encourages you to share your software, code, models, algorithms, protocols, methods and other useful materials related to the project.

Below are a number of ways in which you can associate data with your article or make a statement about the availability of your data when submitting your manuscript. If you are sharing data in one of these ways, you are encouraged to cite the data in your manuscript and reference list. Please refer to the "References" section for more information about data citation. For more information on depositing, sharing and using research data and other relevant research materials, visit the research data page.
Data linking
If you have made your research data available in a data repository, you can link your article directly to the dataset. Elsevier collaborates with a number of repositories to link articles on ScienceDirect with relevant repositories, giving readers access to underlying data that gives them a better understanding of the research described.

There are different ways to link your datasets to your article. When available, you can directly link your dataset to your article by providing the relevant information in the submission system. For more information, visit the database linking page.

For supported data repositories a repository banner will automatically appear next to your published article on ScienceDirect.

In addition, you can link to relevant data or entities through identifiers within the text of your manuscript, using the following format: Database: xxxx (e.g., TAIR: AT1G01020; CCDC: 734053; PDB: 1XFN).

Mendeley Data
This journal supports Mendeley Data, enabling you to deposit any research data (including raw and processed data, video, code, software, algorithms, protocols, and methods) associated with your manuscript in a free-to-use, open access repository. During the submission process, after uploading your manuscript, you will have the

opportunity to upload your relevant datasets directly to *Mendeley Data*. The datasets will be listed and directly accessible to readers next to your published article online. For more information, visit the Mendeley Data for journals page.

Data in Brief
You have the option of converting any or all parts of your supplementary or additional raw data into one or multiple data articles, a new kind of article that houses and describes your data. Data articles ensure that your data is actively reviewed, curated, formatted, indexed, given a DOI and publicly available to all upon publication. You are encouraged to submit your article for *Data in Brief* as an additional item directly alongside the revised version of your manuscript. If your research article is accepted, your data article will automatically be transferred over to *Data in Brief* where it will be editorially reviewed and published in the open access data journal, *Data in Brief*. Please note an open access fee of 500 USD is payable for publication in *Data in Brief*. Full details can be found on the Data in Brief website. Please use this template to write your Data in Brief.

MethodsX
You have the option of converting relevant protocols and methods into one or multiple MethodsX articles, a new kind of article that describes the details of customized research methods. Many researchers spend a significant amount of time on developing methods to fit their specific needs or setting, but often without getting credit for this part of their work. MethodsX, an open access journal, now publishes this information in order to make it searchable, peer reviewed, citable and reproducible. Authors are encouraged to submit their MethodsX article as an additional item directly alongside the revised version of their manuscript. If your research article is accepted, your methods article will automatically be transferred over to MethodsX where it will be editorially reviewed. Please note an open access fee is payable for publication in MethodsX. Full details can be found on the MethodsX website. Please use this template to prepare your MethodsX article.

Data statement
To foster transparency, we encourage you to state the availability of your data in your submission. This may be a requirement of your funding body or institution. If your data is unavailable to access or unsuitable to post, you will have the opportunity to indicate why during the submission process, for example by stating that the research data is confidential. The statement will appear with your published article on ScienceDirect. For more information, visit the Data Statement page.

Submission checklist
The following list will be useful during the final checking of an article prior to sending it to the journal for review. Please consult this Guide for Authors for further details of any item.

Ensure that the following items are present:
One author has been designated as the corresponding author with contact details:
- E-mail address
- Full postal address
- Telephone
-

All necessary files have been uploaded, and contain:

- Keywords
- All figure captions
- All tables (including title, description, footnotes) Further considerations
- Manuscript has been 'spell-checked' and 'grammar-checked'
- All references mentioned in the Reference list are cited in the text, and vice versa
- Permission has been obtained for use of copyrighted material from other sources (including the Web) For any further information please visit our customer support site at http://support.elsevier.com.

AFTER ACCEPTANCE

Online proof correction

Corresponding authors will receive an e-mail with a link to our online proofing system, allowing annotation and correction of proofs online. The environment is similar to MS Word: in addition to editing text, you can also comment on figures/tables and answer questions from the Copy Editor. Web-based proofing provides a faster and less error-prone process by allowing you to directly type your corrections, eliminating the potential introduction of errors.

If preferred, you can still choose to annotate and upload your edits on the PDF version. All instructions for proofing will be given in the e-mail we send to authors, including alternative methods to the online version and PDF.

We will do everything possible to get your article published quickly and accurately. Please use this proof only for checking the typesetting, editing, completeness and correctness of the text, tables and figures. Significant changes to the article as accepted for publication will only be considered at this stage with permission from the Editor. It is important to ensure that all corrections are sent back to us in one communication. Please check carefully before replying, as inclusion of any subsequent corrections cannot be guaranteed. Proofreading is solely your responsibility.

Offprints

The corresponding author will, at no cost, receive a customized Share Link providing 50 days free access to the final published version of the article on ScienceDirect. The Share Link can be used for sharing the article via any communication channel, including email and social media. For an extra charge, paper offprints can be ordered via the offprint order form which is sent once the article is accepted for publication. Both corresponding and co-authors may order offprints at any time via Elsevier's Webshop. Corresponding authors who have published their article gold open access do not receive a Share Link as their final published version of the article is available open access on ScienceDirect and can be shared through the article DOI link.

AUTHOR INQUIRIES

Visit the Elsevier Support Center to find the answers you need. Here you will find everything from Frequently Asked Questions to ways to get in touch.

You can also check the status of your submitted article or find out when your accepted article will be published.