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Flávia Maria Moura de Paula

"Modulação de Peroxirredoxinas em Linhagem de Células Beta Produtoras de Insulina Expostas à Citocinas"

Orientador: Prof. Dr. Antonio Carlos Boschero

Co-Orientador: Prof. Dr. Kléber Luiz de Araujo e Souza

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Flávia Maria Moura de Paula

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Tese apresentada ao Instituto de Biologia para obtenção do Título de Doutora em Biologia Funcional e Molecular, na área de Fisiologia.

Orientador: Prof. Dr. Antonio Carlos Boschiero
Coorientador: Prof. Dr. Kléber Luiz de Araújo e Souza

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Assinatura

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Sumário

LISTA DE ABREVIATURAS E FÓRMULAS MOLECULARES

Lista de Abreviaturas e Fórmulas Moleculares

ANOVA – Analysis of Variance

AKT – v-akt Murine Thymoma Viral Oncogene Homolog 1

AP1 – Activator Protein 1

BB – Biobreeding

$C_6H_{12}O_6$ – Glucose

$CaCl_2.2H_2O$ – Calcium Chloride Dihydrate

$CD3^+$ - Cluster of Differentiation 3

c-Myc – v-myc Myelocytomatosis Viral Oncogene Homolog (avian)

CO_2 – Carbon Dioxide

CuZnSOD – Copper/Zinc Superoxide Dismutase

DM – Diabetes Mellitus

DNA – Deoxyribonucleic Acid

DTT – Dithiothreitol

FADD – Fas-Associated Protein with Death Domain

FasL – Fas Ligand

FBS – Fetal Bovine Serum

FOXO3A – Forkhead Box O3

GAPDH – Glyceraldehyde 3-phosphate Dehydrogenase

GPx – Glutathione Peroxidase

GTP – Guanosine Triphosphate

Hepes – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic Acid

H_2O – Dihydrogen Monoxide

H_2O_2 – Hydrogen Peroxide

HRP – Horseradish Peroxidase

IFN-gamma – Interferon gamma

IFN-R – IFN-gamma Receptor

IkB – Inhibitor of kappa B

IKK – IkappaB Kinase

IL-1 alpha – Interleukin 1 alpha

IL1- beta – Interleukin 1 beta

IL1-R – Interleukin 1 Receptor

IL-2 – Interleukin 2

IL-4 – Interleukin 4

IL-10 – Interleukin 10

IL-13 – Interleukin 13

iNOS – Inducible Nitric Oxide Synthase

INS-1 – Rat Insulinoma 1

IRAK – Interleukin-1 Receptor-associated Kinase

JAK – Janus Kinase

JNK – c-Jun N-terminal Kinase

KCl – Potassium Chloride

KH_2PO_4 – Monopotassium Phosphate

K_2HPO_4 – Dipotassium Hydrogen Phosphate

MAPK – Mitogen-activated Protein Kinase

$MgSO_4$ – Magnesium Sulfate

MnSOD – Manganese Superoxide Dismutase

MTS – 3-*(*4,5-dimethylthiazol-2-yl*)*-5-(*3*-carboxymethoxyphenyl*)*-2-*(*4-sulfophenyl*)*-2H-tetrazolium

MyD88 – Myeloid Differentiation Factor 88

NaCl – Sodium Chloride

$NaHCO_3$ – Sodium Bicarbonate

$Na_2HPO_4.2H_2O$ – Disodium Hydrogen Phosphate

NADPH – Nicotinamide Adenine Dinucleotide Phosphate

NFkB – Nuclear Factor kappa-light-chain-enhancer of Activated B Cells

$NO^{\cdot}$ – Nitric Oxide

NOD – Non-obese Diabetic

O_2 – Oxygen

$O_2^{\cdot -}$ - Superoxide Anion

$^{\cdot}HO$ – Hydroxyl Radical

$ONOO^-$ - Peroxynitrite

P70S6K - p70 Ribosomal Protein S6 Kinase

PBS – Phosphate Buffer Solution

RT-PCR – Reverse Transcriptase Polimerase Chain Reaction

PDTC – Pyrrolidine Dithiocarbamate

PKC – Protein Kinase C

PI3K – Phosphatidylinositol-3-Kinase

PIAS – Protein Inhibitors of Activated STAT

PLC – Phospholipase C

PMS – Phenazine Methosulfate

PRDX – Peroxiredoxin

PTP – Protein Tyrosine Phosphatase

RINm5F – Rat Insulinoma m5F

RNA – Ribonucleic Acid

RNS – Reactive Nitrogen Species

ROS – Reactive Oxygen Species

RPMI1640 – Roswell Park Memorial Institute 1640

SDS-PAGE – Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

S.E.M. – Standard Error of the Mean

siRNA – Small Interfering RNA

SOCS – Suppressor of Cytokine Signaling

STAT1 – Signal Transducers and Activators of Transcription 1

Th – T helper

TNF-alpha – Tumor Necrosis Factor alpha

TNF-beta – Tumor Necrosis Factor beta

TRADD – TNF Receptor Death Domain-associated

TRAF6 – TNF Receptor Associated Factor 6

TRAIL – TNF-related Apoptosis-inducing Ligand

RESUMO

Resumo

Durante a instalação do diabetes tipo 1 as células beta pancreáticas são alvos do ataque pelo sistema de defesa do organismo. A morte das células beta, em geral por apoptose, é provocada por contato direto com células ativadas do sistema imune e por mediadores inflamatórios tais como: citocinas pró-inflamatórias, quemocinas e radicais livres. As citocinas pró-inflamatórias, como IL1-beta, TNF-alpha e IFN-gamma, produzem grande quantidade de ROS e RNS no interior das células beta e estas, por sua vez, possuem uma baixa defesa anti-oxidante enzimática, principalmente ao que se refere às enzimas que degradam H_2O_2, como catalase e glutationa peroxidase. Tal combinação resulta no surgimento de estresse oxidativo e morte celular. Adicionalmente, outro sistema de peroxidases, as PRDXs, também atuam na proteção das células beta contra o estresse oxidativo. Neste sentido, o estudo sobre a modulação de PRDXs por agentes inflamatórios é de grande valia, à medida que se tenta descobrir novas vias intracelulares desencadeadas pelas citocinas e alternativas para suprir a vulnerabilidade das células beta pancreáticas ao estresse oxidativo. Para isso utilizamos linhagem de células beta produtoras de insulina RINm5F. Estas células foram expostas às citocinas pró-inflamatórias IL1-beta, TNF-alpha e IFN-gamma e à anti-inflamatória IL-4 e a expressão das PRDXs foi analizada. Nossos resultados demonstram que IFN-gamma e TNF-alpha reduzem a expressão da PRDX6. Quando separadas, essas citocinas alteram somente a expressão protéica, através da ativação de sistemas de proteólise, especialmente de calpaínas e ubiquitina-proteassomo, e via ativação da JNK/c-Jun. A pré-incubação das células RINm5F com a citocina anti-inflamatória IL4, bloqueia os efeitos do TNF-alpha ou IFN-gamma sobre a expressão da PRDX6. Em conjunto, IFN-gamma e TNF-alpha reduzem tanto a expressão gênica quanto

protéica da PRDX6. As alterações transcricionais ocorrem, provavelmente, por ação sinérgica de mais de uma via intracelular, neste caso, NFkB (ativado pelo TNF-alpha) e STAT1 (ativado pelo IFN-gamma), sendo necessária a participação dessas duas vias para a modulação gênica da PRDX6. A deleção dessa enzima aumenta a suceptibilidade das células RINm5F aos efeitos deletérios de IFN-gamma, TNF-alpha e H_2O_2, sugerindo função importante da PRDX6 na proteção das células beta ao estresse oxidativo.

ABSTRACT

Abstract

Peroxiredoxins are a family of six antioxidant enzymes (PRDX1-6), and may be an alternative system for the pancreatic beta cells to cope with oxidative stress. This study investigated whether the main diabetogenic pro-inflammatory cytokines or the anti-inflammatory cytokine IL-4 modulate PRDXs levels and putative intracellular pathways important for this process in the insulin-producing RINm5F cells. RINm5F cells expressed significant amounts of PRDX1, PRDX3 and PRDX6 enzymes. Only PRDX6 was modulated by cytokines, showing both mRNA and protein down-regulation following incubation of RINm5F cells with TNF-alpha and IFN-gamma but not with IL-1beta. Separately IFN-gamma or TNF-alpha decreased PRDX6 protein but not mRNA levels. The blockage of the JNK signalling and of the calpains and proteasome proteolysis systems restored PRDX6 protein levels. IL-4 alone did not modulate PRDXs levels. However, pre/co-incubation with IL-4 substantially prevented the decrease in PRDX6 induced by pro-inflammatory cytokines. Knockdown of PRDX6 increased susceptibility of RINm5F cells to the deleterious effects of pro-inflammatory cytokines and to oxidative stress. These results show that, from the PRDXs highly expressed in RINm5F cells, only PRDX6 is modulated by the diabetogenic cytokines IFN-gamma and TNF-alpha. This PRDX6 down-regulation depends on the Calpain and proteasome systems and JNK signalling. PRDX6 is an important enzyme for protection against oxidative stress and the interaction between pro- and anti-inflammatory cytokines might be important to determine the antioxidant capacity of the cells.

INTRODUÇÃO

Introdução

Diabetes Mellitus tipo 1 – Citocinas pró-inflamatórias.

O Diabetes Mellitus (DM), doença caracterizada por aumento nas concentrações de glicose plasmática, resulta de defeitos na secreção e/ou ação da insulina, hormônio produzido e secretado pelas células beta pancreáticas, localizadas nas ilhotas de *Langerhans* (Deeney JT, 2000). As duas formas principais da doença são DM1 e DM2. Ambas apresentam falhas progressivas das células beta. No DM1 essas falhas se devem ao ataque direto do sistema imune às células beta de indivíduos geneticamente pré-dispostos, induzindo rápida morte celular. Já o DM2 caracteriza-se por defeitos na ação da insulina nos tecidos periféricos responsivos à este hormônio (resistência à insulina), associados à incapacidade das células beta em produzir quantidade suficiente insulina para compensar essa resistência. Neste caso, a lipotoxicidade (devido à obesidade normalmente associada ao desenvolvimento do DM2) em adição à glicotoxicidade (resultante da resistência periférica à insulina) podem atuar negativamente sob as células beta, diminuindo sua função e massa (Cnop M, 2005).

O conceito atual de DM1 distingue a patologia como multifatorial, em que a associação da pré-disposição genética (polimorfismos) com a exposição à fatores ambientais (microorganismos, substâncias químicas, estado nutricional) resulta em desregulação do sistema imune contra as células beta (Rabinovitch A, 1998). Esse descontrole imunológico promove um desbalanço entre os linfócitos T auxiliadores 1 e 2 (células T *helper*- Th1 e Th2) (Rabinovitch A, 1994). De acordo com essa hipótese, o aumento na expressão e ação de células Th1 e das citocinas por elas liberadas (IL-2, IFN-

gamma e TNF-beta), bem como a diminuição na expressão e ação das células Th2 e suas citocinas (IL-4 e IL-10) leva à ativação de macrófagos e linfócitos T citotóxicos que, que por sua vez, destroem as células beta através de 2 mecanismos: (1) contato direto dos linfócitos T citotóxicos com as células beta (envolvimento de Fas/FasL; perforina; granzima); (2) liberação de mediadores inflamatórios como citocinas (IL1-beta, TNF-alpha, TNF-beta e IFN-gamma) e radicais livres ($O_2^{\cdot-}$, H_2O_2, $NO^{\cdot}$) sobre as células beta (Figura 1) (Rabinovitch A, 1998).

Dentre as citocinas pró-inflamatórias liberadas pelo sistema imune, IL1-beta, TNF-alpha e IFN-gamma são as que provocam maiores danos às células beta. A transdução intracelular do sinal dessas citocinas envolve: (1) ligação das citocinas ao seu receptor específico de membrana; (2) ativação de fosfatases e quinases citosólicas, principalmente quinases mitogênicas e ativadas por estresse; (3) mobilização de fatores de transcrição envolvidos na ativação da morte celular, como NFkB, AP-1, c-Jun, STAT-1; (4) modulação da transcrição de genes relacionados com diferenciação, função e sobrevivência das células beta (Eizirik DL, 2001).

As citocinas sinalizam para as células beta através de diferentes vias intracelulares. A IL1-beta e a IL1-alpha sinalizam através da ligação ao mesmo receptor de membrana (IL1-R). Existem 2 tipos de receptores de IL-1beta (IL1-R1 e IL1-R2) sendo que as células beta expressam ambos os tipos. Após a ligação da IL1-beta ao IL1-R, o domínio citoplasmático do receptor que possui atividade GTPase é ativado, promovendo a hidrólise do GTP, o que desencadeia mudanças na conformação do complexo citocina-receptor. Essas mudanças são necessárias para que proteínas acessórias e adaptadoras como IRAK e MyD88 sejam recrutadas para próximo do complexo (Auron PE, 1998). O recrutamento da

IRAK é essencial para a sinalização da IL1-beta, pois tal proteína interage com TRAF6, implicando em ativação de NFkB via IKK (Eizirik DL, 2001). Após ativação do complexo IKK, degradação de IkB e liberação do NFkB, este fator de transcrição migra para o núcleo e induz a transcrição ou repressão de genes alvos (Eizirik DL, 2001). Além da via do NFkB (principal via de ativação), a IL1-beta também sinaliza através da ativação de MAPKs e PLC-PKC (Figura2).

Outra citocina pró-inflamatória que participa da destruição das células beta durante o DM1 é o IFN-gamma. Esta citocina liga-se ao seu receptor de membrana 1 (IFN-R1), que dimeriza-se recrutando 2 proteínas de membrana acessórias (IFN-R2). A associação de IFN-R1 com IFN-R2 promove a ligação das proteínas JAK1 e JAK2 ao complexo citocina-receptor (Gysemans C, 2008). Ocorre então auto- e trans-fosforilação de JAK1 e JAK2, liberando sítios de ancoragem para 2 moléculas de STAT1. Após ligação, as STATs são fosforiladas pela JAK2, se desligam do complexo, dimerizam-se e translocam-se para o interior do núcleo, ligando-se à regiões específicas do DNA e modulando a transcrição gênica (Eizirik DL, 2001). A ativação da STAT1 é controlada por famílias de reguladores negativos, como PTPs, SOCs e PIASs, sendo principalmente SOCS1 e SOCS3, os inibidores críticos da sinalização do IFN-gamma (Gysemans C, 2008) (Figura 2).

Além de IL1-beta e IFN-gamma, o TNF-alpha também sinaliza para apoptose das células beta. Essa citocina faz parte de uma super-família de 18 membros, incluindo TNF-beta, FasL e TRAIL. Existem 2 tipos de receptores de TNF, o receptor p80 e p60. A isoforma p60 é expressa na maioria dos tecidos enquanto a isoforma p80 é encontrada principalmente em células imunes e endoteliais (Rath PC, 1999). Após ligação do TNF-alpha ao receptor p60, este altera sua conformação e trimeriza-se. O domínio de morte do

receptor ativado é exposto, possibilitando a interação com proteínas acessórias, TRADD e FADD. Este complexo protéico é capaz de ativar diversas quinases como NIK, PKC, JNK, IKK dentre outras, culminando na ativação de 2 fatores de transcrição principais, c-Jun e NFkB, modulação da transcrição gênica e conseqüente morte celular (Eizirik DL, 2001) (Figura2).

Modelos animais de DM1, como camundongos NOD e ratos BB (caracterizados pela presença de auto-anticorpos específicos para as células beta e linfócitos reativos), apresentam altas concentrações de citocinas pró-inflamatórias no micro-ambiente da ilhota pancreática durante os estágios iniciais de insulite (Sigfrid LA, 2004), confirmando a atuação dessas moléculas sobre a morte das células beta (Pirot P, 2008). Sabe-se que as citocinas pró-inflamatórias agem de forma sinérgica, e quando em conjunto, tornam as células beta mais suceptíveis à morte celular, principalmente por apoptose (Eizirik DL, 2001; Pirot P, 2008; Souza KL, 2008). Contrariamente, as citocinas anti-inflamatórias, em especial IL-4, IL-10 e IL-13 são capazes de prevenir muitos dos efeitos danosos da IL1-beta às células beta, por exemplo, reduzindo a produção de $NO^{\cdot}$ e aumentando a proteção celular (Perretti M, 1995; Souza KL, 2008).

Atualmente, sugere-se a participação de estresse nitro-oxidativo e de retículo endoplasmático, bem como mediadores mitocondriais (citocromo c; caspase-9) e não-mitocondriais (NADPH oxidase), como os principais mecanismos intracelulares indutores de morte celular, desencadeados pelas citocinas pró-inflamatórias (Cnop M, 2005; Morgan D, 2007; Pirot P, 2008; Souza KL, 2008; Gurgul-Convey E, 2011). Já as citocinas anti-inflamatórias sinalizam através da ativação de vias de sobrevivência e proliferação celular,

como PI3K/AKT e MAPKs e diminuição das vias de morte celular (Nishisaka F, 2001; Kaminski A, 2007; Souza KL, 2008; Kaminski A, 2009).

Estresse Oxidativo – Enzimas Anti-oxidantes - Peroxirredoxinas.

A cito-toxicidade mediada pela ação das citocinas pró-inflamatórias é decorrente do aumento na produção de ROS e RNS. Este desbalanço no estado redox aumenta a peroxidação lipídica, oxidação protéica e danos à molécula de DNA o que, em última instância, leva a morte das células beta (Cnop M, 2005; Lenzen S, 2008). Na presença de citocinas pró-inflamatórias, as células beta aumentam a geração de ROS ($O^{\cdot-}_2$, H_2O_2 e $^{\cdot}HO$), uma vez que tais citocinas, induzem o aumento na expressão de NADPH oxidase, complexo enzimático localizado na membrana plasmática (Oliveira HR, 2003; Morgan D, 2007; Newsholme P, 2007), que produz $O^{\cdot-}_2$ a partir de equivalentes redutores (NADPH) e oxigênio molecular (O_2). Além disso, as citocinas atuam sobre as mitocôndria, aumentando a formação de $O^{\cdot-}_2$ e modificando o potencial de membrana mitocondrial, o que promove liberação de citocromo c para o citosol, ativação de caspase-9 e -3 e, morte celular (Newmeyer DD, 2003; Cnop M, 2005). Adicionalmente, as citocinas pró-inflamatórias, em especial IL-1beta e TNF-alpha, por ativarem NFkB, induzem a transcrição de genes resposivos à esse fator de transcrição, como iNOS e MnSOD (Cnop M, 2005; Souza KL, 2008). A ativação de iNOS leva a um aumento na produção de $NO^{\cdot}$ que, ao se juntar com $O^{\cdot-}_2$ (aumentado pela ativação de NADPH oxidase e fosforilação oxidativa), forma $ONOO^-$, altamente tóxico para as células beta (Gurgul-Convey E, 2011). Já a expressão aumentada da enzima mitocondrial MnSOD e a baixa expressão das peroxidases catalase e GPx,

acumulam H_2O_2 especialmente próximo a mitocôndria que, juntamente com $NO^{\cdot}$, forma $^{\cdot}HO$, causando danos à esta organela (Lenzen S, 2008; Gurgul-Convey E, 2011) (Figura 3).

O desbalanço no estado redox, provocado pela ação das citocinas pró-inflamatórias, é particularmente danoso às células beta, pois a capacidade de detoxificação enzimática destas células em roedores é bastante limitada, tornando-as vulneráveis ao acúmulo de ROS e RNS (Lenzen S, 2008).

Quando comparada a outros tecidos (hepático, muscular, nervoso, renal), as células beta expressam baixa quantidade das principais enzimas anti-oxidantes, sendo deficiente principalmente em enzimas que convertem H_2O_2 em H_2O, como GPx e catalase (Grankvist K, 1981; Lenzen S, 1996; Tiedge M, 1997). Neste sentido, estudos *in vitro* demonstraram que o aumento na defesa antioxidante das células beta pela superexpressão de catalase e GPx, separadamente e principalmente em conjunto, previne a cito-toxicidade induzida por citocinas pró-inflamatórias e H_2O_2 (Tiedge M, 1998; Lortz S, 2000). Ainda, a superexpressão de catalase restrita à mitocôndria proporciona melhor proteção das células beta à esses agentes cito-tóxicos (Gurgul E, 2004; Gurgul-Convey E, 2011).

Além das enzimas catalase e GPx, as células beta possuem um outro sistema de degradação de H_2O_2, as peroxirredoxinas (PRDXs). As PRDXs compõem uma família de enzimas anti-oxidantes, thiol-específicas. Exercem seus efeitos por sua atividade peroxidase ($ROOH + 2e^- \rightarrow ROH + H_2O$), oxidando tanto tiorredoxinas quanto glutationa, na qual, H_2O_2, $ONOO^-$ e vários tipos de hidroxiperóxidos orgânicos são reduzidos (Bryk R, 2000; Peshenko IV, 2001). Seis isoformas de PRDXs foram identificadas em células de mamíferos distribuídas na maioria dos compartimentos celulares, cobrindo todos os locais possíveis de formação de ROS. As PRDX1, PRDX2 e PRDX6 estão localizadas no citosol,

a PRDX3 é restrita à mitocôndria, a PRDX4 está localizada no retículo endoplasmático e também pode ser secretada para o meio extracelular e a PRDX5 está localizada na mitocôndria e no peroxissomo (Wood ZA, 2003). As PRDXs também são divididas de acordo com a posição molecular e o número de resíduos de cisteína que possuem atividade peroxidase e são ativados durante a reação de oxi-redução (Wood ZA, 2003).

Pouco se sabe a respeito das PRDXs nas células beta. Contudo, já foi demonstrado que células INS1 respondem a IL1-beta aumentando a expressão de PRDX1 e PRDX2 e isso poderia ser resultado da produção intracelular aumentada de $NO^{\cdot}$ e/ou H_2O_2 em resposta a IL1-beta (Bast A, 2002). Interessante notar que o aumento de MnSOD pode estar associado a uma diminuição de PRDX1, e essa combinação de fatores pode determinar se a célula entrará em apoptose (Han YH, 2005).

Foi demonstrado que os fatores de transcrição Forkhead Box Protein O3a (FOXO3A) (Chiribau CB, 2008) e c-Myc (Wonsey DR, 2002) são os principais moduladores da expressão gênica da PRDX3. Tal indício torna essa enzima interessante para estudos em modelos de diabetes, uma vez que há evidências de que a desregulação de genes da família Forkhead-box está envolvida nessa síndrome (Katoh M, 2004; Glauser DA, 2007). Além de seu efeito peroxidase (Chang TS, 2004), a PRDX3 também modula a função secretória das células beta, uma vez que a diminuição na expressão de PRDX3 em células RINm5F diminui a secreção de insulina basal e estimulada por glicose (Wolf G, 2010).

A PRDX4, primeiramente identificada em células testiculares (Matsuki S, 2002), diminuie a ativação de NFkB, especificamente diminuindo a fosforilação de IkB-α e conseqüentemente a migração do dímero p50/p65 do NFkB para o núcleo (Jin DY, 1997).

Tal evidência indica que as PRDXs também podem modificar a resposta das células beta contra compostos citotóxicos que ativem essa via como, IL1-beta e TNF-alpha. A PRDX4 também é expressa em ilhotas pancreáticas humanas (Ahmed M, 2005) e sua superexpressão reduz a infiltração de linfócitos CD3-positivos para o interior da ilhota, reprime a apoptose induzida por estreptozotocina e, aumenta a taxa de proliferação de células beta (Ding Y, 2010). Tais mecanismos se dão pela diminuição na ativação das vias de NFkB e STAT1, diminuição na expressão de IL1-beta e TNF-alpha, bem como de seus receptores, sugerindo um papel essencial da PRDX4 no balanço redox intracelular e sobre o desenvolvimento do DM1 (Ding Y, 2010).

Embora descrita, recentmente, em mamíferos, a PRDX5 foi a isoforma mais estudada quanto à proteção celular ao estresse oxidativo (Plaisant F, 2003; Tiên Nguyên-nhu N, 2003; Banmeyer I, 2004; Dubuisson M, 2004; Banmeyer I, 2005). Trata-se de uma proteína presente principalmente em mitocôndrias e peroxissomos (Wood ZA, 2003). A superexpressão da PRDX5 na mitocrôndria, (Banmeyer I, 2004; 2005), mostrou-se uma estratégia eficiente para proteger células de ovário à ação deletéria do H_2O_2. Até o momento, não existem estudos dessa isoforma em ilhotas pancreáticas.

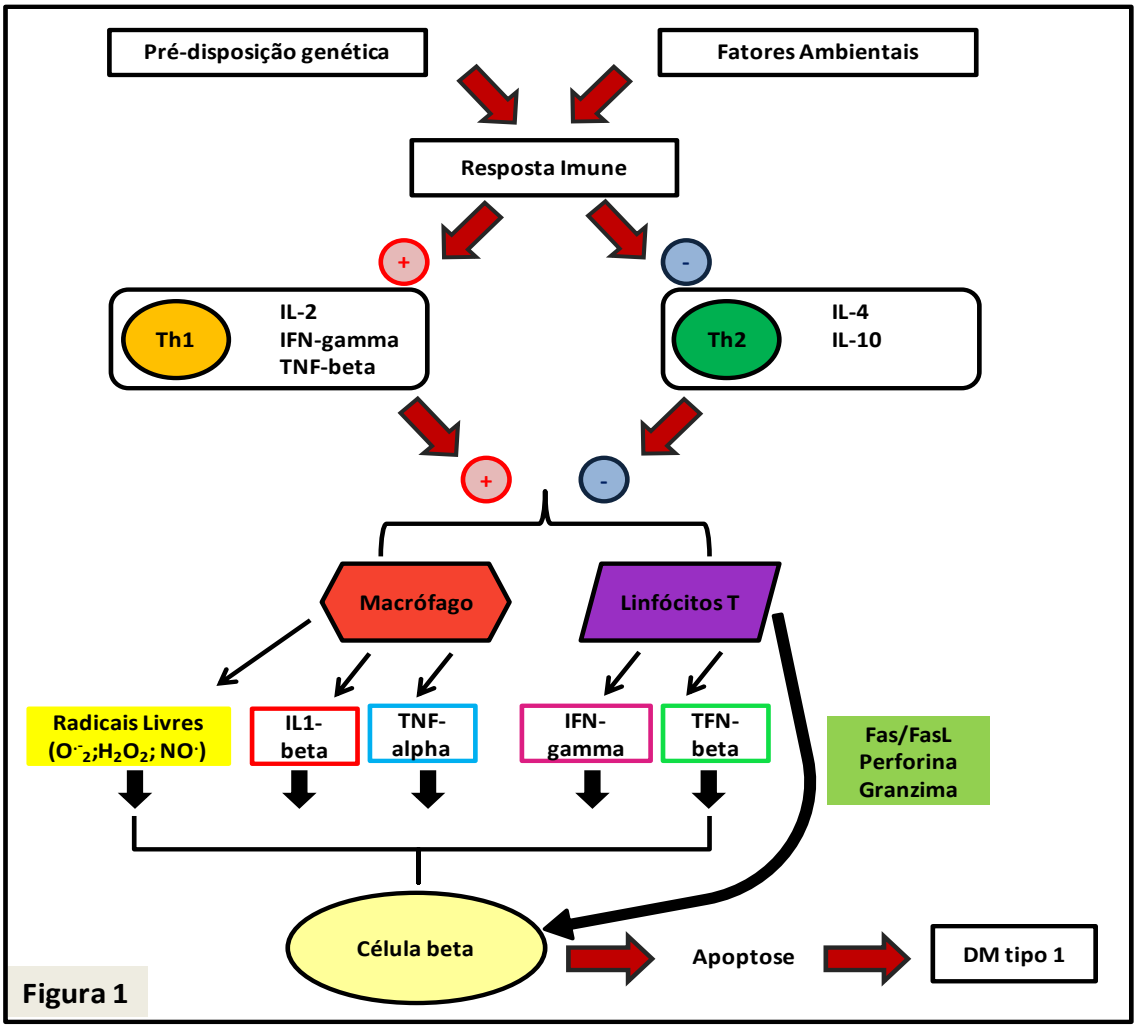
Por último, a PRDX6, proteína amplamente distribuída no meio intracelular, confere proteção as células contra os danos causados por altas concentrações de oxigênio, indicando uma função primariamente anti-oxidante (Wang Y, 2006b). Em concordância, a sua deleção, aumenta a sensibilidade de células pulmonares ao estresse oxidativo (Wang Y, 2006a). Interessantemente, altas concentrações de glicose aumentam a expressão de PRDX6 em células mesangiais (Morrison J, 2004), efeito esse que poderia ocorrer também em células beta de indivíduos diabéticos tipo 2. A expressão da PRDX6 foi confirmada em

ilhotas pancreáticas (Nagaoka Y, 2004), no entanto, sua função e modulação nas células beta são pouco compreendidas.

De um modo geral, pouco se sabe sobre a função das PRDXs nas células beta pancreáticas submetidas ao estresse oxidativo e morte celular. Sugere-se que tais enzimas tenham uma função importante na proteção celular durante a instalação do diabetes. Entretanto, a modulação da expressão das isoformas de PRDXs em células beta pancreáticas, não foi criteriosamente avaliada.

A exposição às citocinas pró-inflamatórias IFN-gamma, IL1-beta e TNF-alpha reproduz de forma muito semelhante às respostas celulares frente o desenvolvimento do DM1, levando a apoptose de células beta (Cnop M, 2005; Souza KL, 2008; Ortis F, 2010). Neste contexto, o estudo sobre a modulação das PRDXs em células beta pancreáticas, incubadas na presença de citocinas pró e anti-inflamatórias, torna-se de grande importância à medida que se relaciona as variações no estado redox intracelular, provocado pela exposição às citocinas, com a modulação na expressão das PRDXs e sua funcionalidade intracelular.

Figuras – Introdução



Mecanismos imunológicos responsáveis pelo desenvolvimento do Diabetes tipo 1. Pré-disposição genética e exposição à fatores ambientais direcionam a resposta imune para proteção ou patogeneicidade. Durante a instalação do DM tipo 1, as células beta são destruídas pela ativação de células T autoreativas (Th1) e suas citocinas que, por sua vez, ativam macrófagos e linfócitos T citotóxicos. Essas células, quando ativas, destróem diretamente as células beta através de 2 mecanismos: contato direto de linfócitos e macrófagos e/ou ação de mediadores inflamatórios como radicais livres e citocinas pró-inflamatórias nas células beta. Adaptado de Rabinovitch, A., 1998.

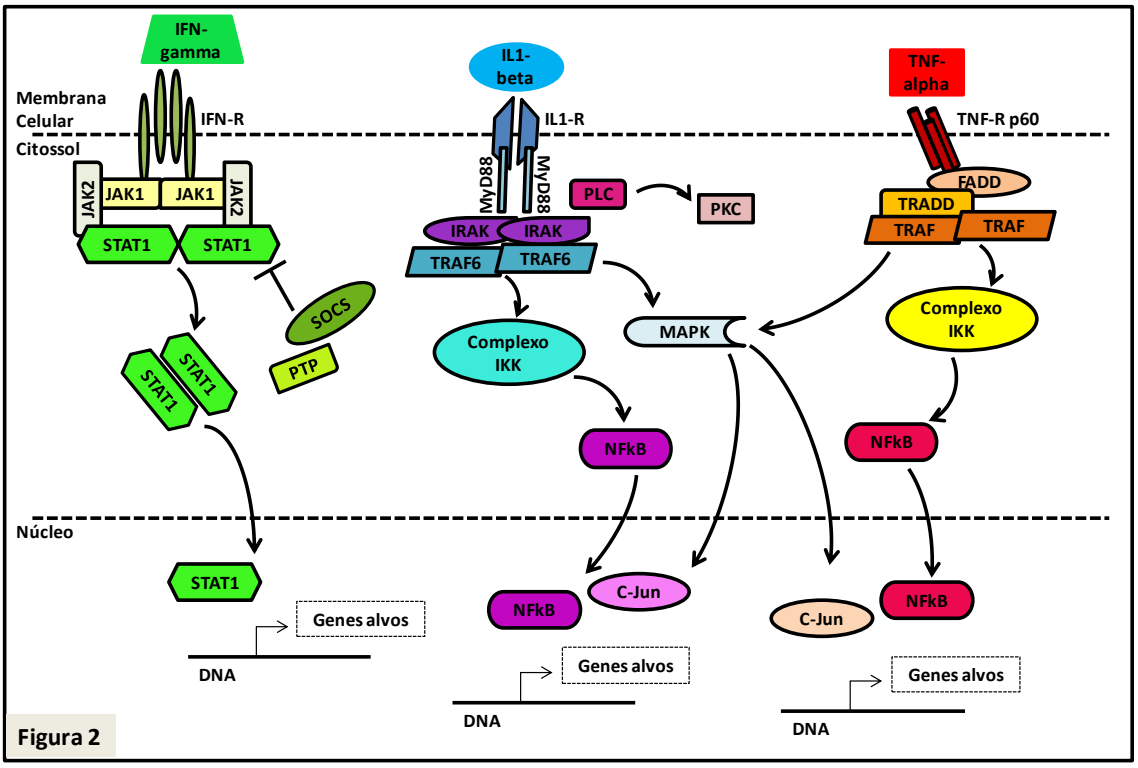


Figura 2

Mecanismos de sinalização intracelular das citocinas pró-inflamatórias IFN-gamma, IL1-beta e TFN-alpha. IFN-gamma sinaliza para as células beta principalmente pela ativação das proteínas JAK e fatores de transcrição STAT1. A IL1-beta e o TNF-alpha modulam, principalmente, vias intracelulares que resultam na ativação do fator de transcrição NFkB porém, outras quinases são ativadas, como MAPKs, levando a ativação outros fatores de transcrição, como c-Jun. Tais mecanismos promovem alterações na transcrição de diversos genes relacionados com proteção, função e sobrevivência das células beta. O *cross-talk* dessas 3 vias de sinalização resulta em morte de células beta, principalmete por apoptose, durante o desenvolvimento do DM tipo 1. Adaptado de Eizirik, DL., 2001.

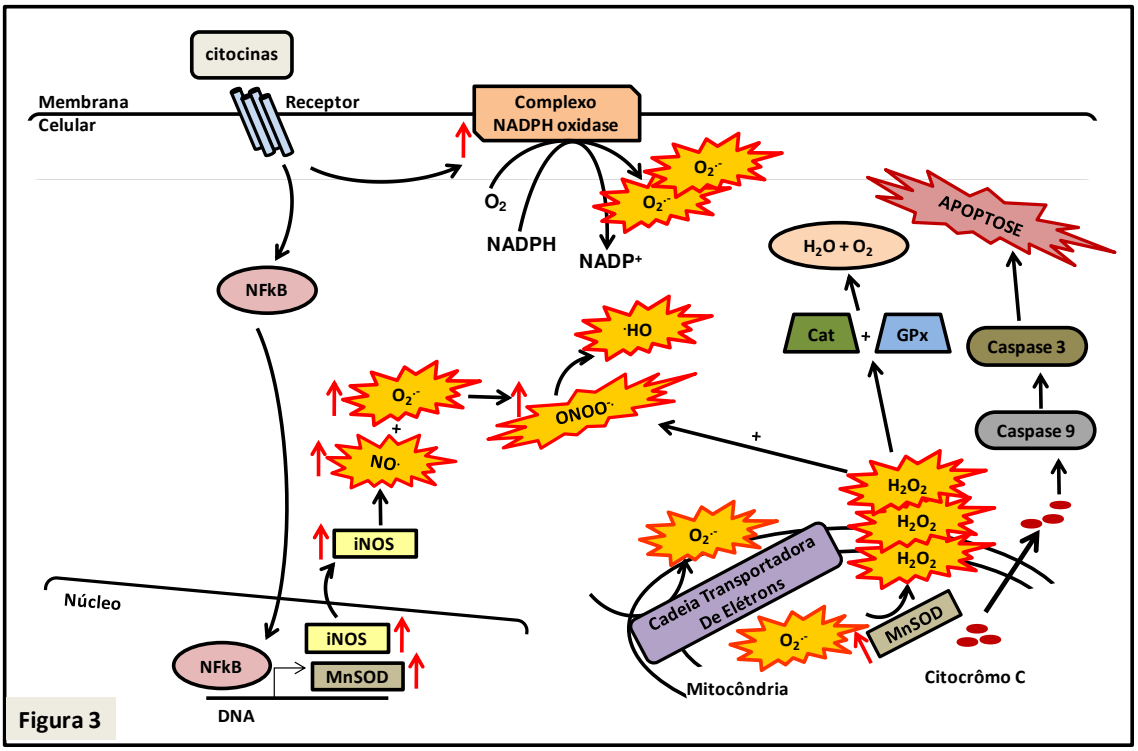


Figura 3

Sitios de formação de espécies reativas de oxigênio e nitrogênio nas células beta após exposição à citocinas pró-inflamatórias. Após ativação de NFkB, este migra para o núcleo e modula a trancrição de genes alvos, como MnSOD e iNOS. A ativação de iNOS aumenta a produção de $NO^{\cdot}$ que, juntamente com o $O_2^{\cdot-}$ gerado tanto pela mitocôndria quanto pela NADPH oxidase, forma $ONOO^{-}$. A MnSOD, por sua vez, ao desmutar o $O_2^{\cdot-}$, gera H_2O_2, que se acumula no interior da célula devido a baixa quantidade de enzimas que o degradam, como catalase e GPx. As citocinas pró-inflamatórias também liberam citocrômo c da mitocôndria, que no citossol, ativa caspases culminando em apoptose das células beta. Adaptado de Newsholme, P., 2007.

OBJETIVOS

Objetivos

Os objetivos desse trabalho foram investigar:

- Se citocinas pró- e anti-inflamatórias modulavam a expressão das PRDXs em linhagem de célula beta RINm5F;

- Quais vias intracelulares estariam envolvidas na modulação das PRDXs;

- Se existia interação entre a sinalização induzida pelas citocinas pró- e anti-inflamatórias na modulação das PRDXs;

- Se a modulação na expressão das PRDXs alterava a viabilidade celular.

Para isso, avaliamos:

-Expressão gênica e protéica de todas as isoformas de PRDXs em células RINm5F incubadas por diferentes períodos de incubação e combinações de citocinas pró-inflamatórias IFN-gamma, TNF-alpha e IL1-beta;

-Expressão gênica e protéica de todas as isoformas de PRDXs em diferentes concentrações e períodos de incubação com a citocina anti-inflamatória IL-4;

- *Cross-talk* entre as vias de sinalização intracelulares envolvidas na modulação das PRDXs por parte das citocinas pró- e anti-inflamatórias;

- Função das PRDXs moduladas pelas citocinas na viabilidade celular;

MATERIAL E MÉTODOS

Material e Métodos

1) Materiais: As citocinas IFN-gamma, IL1-beta e TNF-alpha recombinantes de rato, Lipofectamina 2000 e siRNA para JNK (Stealth Select siRNA Duplex Oligoribonucleotides) foram adquiridas da Invitrogen Corp. (Carlsbad, CA, USA) e a citocina IL-4 recombinante de rato da Thermo Scientific (Miami, OK, USA). siRNA para PRDX6 (Silencer Select Pre-designed siRNA) foi adiquirido da Life Technologies do Brasi LTDA., (São Paulo, Brazil). Todos os reagente de SDS-PAGE e imunobloting foram obtidos da BioRad (Richmond, CA, USA). Anticorpos anti-PRDX1, anti-PRDX2, anti-PRDX3, anti-PRDX4, anti-PRDX5 e anti-PRDX6 (Abcam Inc. Cambridge, MA, USA), anti-GAPDH (Santa Cruz Biotechnology, CA, USA) e anti-phospho c-JUN ser63 e anti-phospho c-JUN ser73 (Cell Signaling Technology, Beverly, MA, USA) foram utilizados para detecção protéica. Meio RPMI1640, outros reagentes de cultivo celular e os inibidores farmacológicos de calpaína (N-Acetyl-L-leucyl-L-leucyl-L-norleucinal, A6185), de JNK (SP600125), de STAT1 (curcumin, 08511) e da subunidade p65 do NFkB (pirrolidina ditiocarbamato, PDTC) foram adquiridos da Sigma (St. Louis, MO, USA). O inibidor de proteassomo (MG132) foi obtido da Enogene Biothec Co (New York, NY, USA) e os reagentes para MTS, da Promega (Madison, WI, USA). Os *primers* foram desenhados com o auxilio do programa Vector NTI Advance™ 11 e as seqüências adquiridas da IDT (Coralville, IA, USA) estão dispostas na Tabela 1. O Sybr Green Master Mix e demais reagentes para RT-PCR em tempo real foram obtidos da Applied Biosystems (Foster City, CA).

***2) Cultura celular e exposição a compostos citotóxicos*:** Células produtoras de insulina RINm5F com passagem entre 80-90, foram cultivadas em meio RPMI 1640, suplementado com 10 mmol/L de glicose, 10 % (v/v) de soro fetal bovino (FBS), 1% de antibióticos (penicilina/ estreptomicina) e anfotericina em atmosfera humidificada a 37 °C e 5 % de CO_2. As células foram plaqueadas e cultivadas até 80% de confluência. A exposição às citocinas foi feita em meio RPMI 1640. Foram utilizadas 14 e 140 U/ml de IFN-gamma, 60 e 600 U/ml de IL1-beta, 185 e 1850 U/ml de TNF-alpha e 2, 20 e 50 ηg/ml de IL4 por 6 e 24 horas. A exposição à H_2O_2 nas concentrações de 25, 50, 100 e 200 μM foi feita em meio livre de FBS e antibióticos. Após 2 horas de incubação com H_2O_2, foram adicionados ao meio de cultura, FBS e antibióticos e a viabilidade celular foi medida ao final de 22 horas. Quando necessário, os inibidores farmacológicos ou siRNAs foram previamente adicionados ao meio de cultura e, posteriormente, as células foram coletadas para os estudos. Células controle foram mantidas em meio RPMI1640 sem adição dos compostos ensaiados.

3) Transfecção do siRNA para JNK e PRDX6: Células RINm5F, semeadas em placas de 24 poços, foram transfectadas usando Lipofectamina 2000, nas concentrações finais de 100 e 200 nmol/L de siRNA para JNK e para PRDX6, de acordo com as instruções do fabricante. As sequências utilizadas foram AAAGAATGTCCTACCTTCT (sense - siJNK) e AGAAGGTAGGACATTCTTT (anti-sense - siJNK); GGAAACCUCAGGUCUUGUAtt(sense - siPRDX6) e UACAAGACCUGAGGUUUCCtc (anti-sense – siPRDX6). Testes utilizando sequência inespecífica de siRNA foram

realizados em pré-experimentos como controle dos experimentos seguintes. Em nenhuma das condições utilizadas nos experimentos, o siRNA inespecífico modulou a expressão da PRDX6, sendo assim, transfecções *mock* foram utilizadas como condição controle. Após transfecção e exposição às citocinas, as células foram lisadas e utilizadas para expressão proteica de PRDX6.

***4) Extração do RNA e PCR em tempo real*:** As amostras foram homogeneizadas em 1 ml de TRIzol (InVitrogen, São Paulo, Brasil) por 1 min. O RNA total foi extraído segundo as instruções contidas no manual do fabricante e quantificado por espectrofotometria. A integridade e pureza do RNA foram visualizadas em gel de agarose. O DNA complementar (cDNA) foi preparado utilizando-se 3 μg de RNA total e reagentes do High Capacity cDNA Transcription Kit da Applied Biosystems (Foster City, CA). O gene GAPDH foi usado como controle endógeno. O PCR em tempo real foi feito em termociclador StepOne (Applied Biosystems) As condições de termociclagem foram 95°C por 10min, seguido de 40 ciclos a 95°C por 10s e 60°C por 30s. As curvas foram analisadas utilizando-se o Sequence Detector System 1.7 (Applied Biosystems). Os resultados foram apresentados como % do controle.

***5) Western blotting*:** Células RINm5F foram coletadas com auxilio de "scraper" e homogeneizadas por sonicação em tampão contendo 8 mol/L uréia (VirSoni 60). Os extratos foram centrifugados a 12000g × 15 min à 4°C para remoção do material insolúvel. Foi adicionado às alíquotas do sobrenadante tampão Laemmli contendo 10 mmol/L de DTT e aquecidas em *heating blocker* a 100°C por 5 min. Alíquotas de 30 μg de extrato protéico

total para PRDX1, PRDX3, PRDX6 e p-cJUN e alíquotas de 50 µg para PRDX2, PRDX4 e PRDX5 foram aplicadas em gel SDS-PAGE (10% Tris-acrilamida) em aparelho minigel (Miniprotean), paralelo com marcador de peso molecular conhecido. Após a corrida, as proteínas foram transferidas para membrana de nitrocelulose. Esta foi incubada por 2 horas em temperatura ambiente em solução bloqueadora contendo 5% de leite desnatado. A seguir as membranas foram incubadas *overnight* com os anticorpos específicos, anti-PRDX1, anti-PRDX6, anti-p-cJUN ser63 e anti-p-cJUN ser73 (1:1000 diluição); anti-PRDX2, anti-PRDX4 e anti-PRDX5 (1:500 diluição) e anti-PRDX3 (1:300 diluição). Anti-GAPDH (1:1000 diluição) foi usado como controle endógeno. Posteriormente, as membranas foram expostas à 150 ng/ml de anticorpo secundário específico conjugado com peroxidase (anti IgG (H+L)-HRP, Invitrogen) por 2 horas em temperatura ambiente. As bandas foram visualizadas por quimiluminescência (SuperSignal, Pierce Biotechnology Inc, Rockford, IL, USA) e quantificadas em programa específico (Scion Corp., Frederick, MD, USA).

***6) Ensaio de viabilidade celular por MTS*:** Células RINm5F foram expostas ao inibidor de calpaína, IFN-gamma, TNF-alpha e H_2O_2 em placas de cultura de 96 poços para estimação da toxicidade desses componetes. Posteriormente, as células foram lavadas com solução de PBS estéril (NaCl 140 mmol/L, KCl 2,6 mmol/L, KH_2PO_4 1,4 mmol/L, $Na_2HPO_4.2H_2O$ 8,1 mmol/L) e incubadas por 4 h em solução de MTS. A viabilidade celular, representada pela produção de NAD(P)H, foi avaliada através do método espectrofotométrico de redução do sal tetrazólio para formazana solúvel (Promega, Madison, USA). A mistura PMS/MTS foi preparada segundo as indicações do fabricante e diluída para 10% em tampão Krebs (NaCl

109 mmol/L, KCl 4,7 mmol/L, $CaCl_2.2H_2O$ 1,9 mmol/L, $MgSO_4$ 1,2 mmol/L, K_2HPO_4 1,03 mmol/L, $NaHCO_3$ 25 mmol/L, Hepes 20 mmol/L, $C_6H_{12}O_6$ 11,1 mmol/L). Os resultados foram apresentados como % da absorbância do MTS em 490 ηm dos grupos controle.

***7) Análises estatísticas*:** Os resultados foram expressos como média ± erro padrão da média (S.E.M.). As comparações entre grupos foram realizadas por One-way e Two-way ANOVA, seguido de teste de Dunnett ou Bonferroni. $p < 0.05$ foi considerado estatisticamente diferente.

RESULTADOS E DISCUSSÃO

Resultados e Discussão

Parte dos resultados obtidos durante a realização deste trabalho estão apresentados a seguir sob a forma de artigo científico publicado na revista 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.

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Abstract

Peroxiredoxins are a family of six antioxidant enzymes (PRDX1-6), and may be an alternative system for the pancreatic beta cells to cope with oxidative stress. This study investigated whether the main diabetogenic pro-inflammatory cytokines or the anti-inflammatory cytokine IL-4 modulate PRDXs levels and putative intracellular pathways important for this process in the insulin-producing RINm5F cells. RINm5F cells expressed significant amounts of PRDX1, PRDX3 and PRDX6 enzymes. Only PRDX6 was modulated by cytokines, showing both mRNA and protein down-regulation following incubation of RINm5F cells with TNF-alpha and IFN-gamma but not with IL-1beta. Separately IFN-gamma or TNF-alpha decreased PRDX6 protein but not mRNA levels. The blockage of the JNK signalling and of the calpains and proteasome proteolysis systems restored PRDX6 protein levels. IL-4 alone did not modulate PRDXs levels. However, pre/co-incubation with IL-4 substantially prevented the decrease in PRDX6 induced by pro-inflammatory cytokines. Knockdown of PRDX6 increased susceptibility of RINm5F cells to the deleterious effects of pro-inflammatory cytokines and to oxidative stress. These results show that, from the PRDXs highly expressed in RINm5F cells, only PRDX6 is modulated by the diabetogenic cytokines IFN-gamma and TNF-alpha. This PRDX6 down-regulation depends on the Calpain and proteasome systems and JNK signalling. PRDX6 is an important enzyme for protection against oxidative stress and the interaction between pro- and anti-inflammatory cytokines might be important to determine the antioxidant capacity of the cells.

Keywords*: Hydrogen peroxide; Diabetes; Gene expression regulation; PRX6; Proteolysis; ROS.*

Abbreviations: Akt, v-akt murine thymona viral oncogene homolog 1; IFN-gamma, interferon gamma; IL-1beta, interleukin 1 beta; JNK, c-Jun N-terminal kinase; NF-kappaB, Nuclear factor of kappa light polypeptide gene enhancer in B cells 1; PI3K, phosphatidylinositol 3'kinase; RNS, reactive nitrogen species; ROS, reactive oxygen species; STAT1, Signal Transducer and Activator of Transcription 1; TNF-alpha, tumour necrosis factor alpha.

1. Introduction

Type 1 Diabetes is a multifactorial disease characterized by chronic inflammation and beta cell loss (Lenzen, 2008; Moore, et al., 2011; Pirot, et al., 2008). Pro-inflammatory cytokines, such as IL-1beta, TNF-alpha and IFN-gamma induce the generation of reactive oxygen and nitrogen species (ROS and RNS) by pancreatic beta cells, leading ultimately to beta cell death (Lenzen, 2008; Souza, et al., 2008). This occurs due to the vulnerability of beta cells to oxidative stress, owing to their low antioxidant capacity. Beta cells express low amounts of both glutathione peroxidase and particularly catalase, enzymes in charge of converting hydrogen peroxide (H_2O_2) into water (H_2O) (Lenzen, 2008; Lenzen, et al., 1996).

Therefore, it may be hypothesized that beta cells may have evolved to use additional systems to get rid of H_2O_2 and other related peroxide compounds. Peroxiredoxins (PRDXs) are a new family of thiol-specific antioxidant proteins that exert their effects via their peroxidase activity ($ROOH + 2e^- \rightarrow ROH + H_2O$), whereby H_2O_2, peroxynitrite and a wide range of organic hydroperoxides are reduced and detoxified (Peshenko and Shichi, 2001). At least six PRDXs were identified in mammalian cells and distributed in most cell compartments. PRDX1, 2 and 6 are localised particularly in the cytosol, PRDX3 is expressed mainly in mitochondria, PRDX4 is located in endoplasmic reticulum and also secreted extracellularly and PRDX5 is located in mitochondria and peroxisomes (Wood, et al., 2003). PRDXs are divided into 3 classes: PRDX1 to 4 are the typical 2-Cys subgroup and exhibit two conserved motifs centred on Cys residues, one in the C-terminus and one in the N-terminus; PRDX5 differs because its C-terminus cysteine is not in the conserved position and PRDX6 conserves only the N-terminus cysteine (Wood et al., 2003).

It has already been shown that pro-inflammatory cytokines have a more hazardous effect on pancreatic beta cells and insulin-producing beta cells when they act in combination (Souza et al., 2008). The cytokine toxicity is partly due to the marked production of nitric oxide (NO) through induction of inducible nitric oxide synthase (iNOS) by the beta cells, particularly when cells are exposed to IL-1beta (Souza et al., 2008). However, there is opposing data suggesting that beta cell damage induced by pro-inflammatory cytokines is not related to the iNOS pathway (Todaro, et al., 2003), and possibly in this case cytotoxicity is due to oxidative stress only, with no participation of nitrosative stress. Pro-inflammatory cytokines also modulate apoptotic pathways, the expression and/or activity of antioxidant enzymes and transcription factors such as caspase-3, manganese superoxide dismutase (MnSOD), catalase, signal transducer and activator of transcription 1 (STAT1), and nuclear factor kappa B (NF-kappaB) (Moore et al., 2011; Ortis, et al., 2010; Souza et al., 2008). In contrast, anti-inflammatory cytokines are able to counteract many of the effects of IL-1beta, reducing NO production and granting cellular protection (Kaminski, et al., 2007; Kaminski, et al., 2010; Souza et al., 2008).

The aim of the present study was to investigate whether pro-inflammatory cytokines modulate the PRDX system at mRNA and protein levels and whether there exists any interaction between anti- and pro-inflammatory cytokines in this modulation in insulin-producing RINm5F cells. We demonstrated that IFN-gamma and TNF-alpha strongly reduce PRDX6 levels, while all other PRDXs are not modulated by cytokines. Activation of Calpain, c-Jun N-terminal kinase (JNK) and proteasome pathways were also major features of RINm5F cells exposed to pro-

inflammatory cytokines. Remarkably, IL-4 was able to reverse these effects. We suggest that the decrease in the PRDX6 levels could contribute to the mechanism of pro-inflammatory cytokine-dependent cytotoxicity in insulin-producing cells in the specific situation of IFN-gamma/TNF-alpha attack.

2. Methods

2.1. Materials

Recombinant rat IFN-gamma, TNF-alpha and IL-1beta cytokines were from Invitrogen Corp (Carlsbad, CA, USA). Recombinant rat IL-4 was from Thermo Scientific (Miami, OK, USA). All SDS-PAGE and immunoblotting equipment's were from Bio-Rad systems (Richmond, CA, USA). Anti-PRDX1, anti-PRDX2, anti-PRDX3, anti-PRDX4, anti-PRDX5 and anti-PRDX6 were from Abcam Inc. (Cambridge, MA, USA). Anti-GAPDH was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Anti-phospho c-Jun ser 63 and anti-phospho c-Jun ser 73 were from Cell Signalling Technology (Beverly, MA, USA). Calpain (N-Acetyl-L-leucyl-L-leucyl-L-norleucinal, catalogue number A6185), JNK (SP600125, c. number S5567), NF-kappaB (ammonium pyrrolidine dithiocarbamate, PDTC, c. number P8765), and STAT1 (curcumin, c. number 08511) inhibitors, RPMI 1640 medium and other culture reagents were from Sigma Chemicals Co (St. Louis, MO, USA). MTS reagent was from Promega (Madison, WI, USA). Proteasome inhibitor (MG132, catalogue number E1B1748) was from Enogene Biotech Co. (New York, NY, USA). SYBR Green Master Mix and other PCR reagents were from Applied Biosystems (Foster City, CA, USA). PCR primers were acquired from IDT (Coralville, IA, USA) and the sequences are presented in supplementary Table S1.

2.2. Cell Culture

RINm5F cells at passages 80-90 were cultured in RPMI 1640 medium, supplemented with 10 mmol/L glucose, 10% foetal calf serum, 1% penicillin and streptomycin in a humidified atmosphere at 37°C and 5% CO_2. Cells were plated and cultured until 80% confluence. Subsequently, cells were exposed to 14 or 140 U/mL of IFN-gamma, 185 or 1850 U/mL of TNF-alpha, 60 or 600 U/mL of IL-1beta and 2, 20 or 50 ng/mL of IL-4. Exposure to hydrogen peroxide was made in antibiotic-free/serum-free medium. After 2 h H_2O_2 incubation, antibiotics (1%) and serum (final concentration of 10%) were added and viability measured 22 h later. When required, inhibitors or PRDX6 or JNK1 siRNA were added to the medium. Control cells were grown in RPMI 1640 medium without addition of test compounds.

2.3. Western-Blotting

Control and cytokine-incubated cells were homogenized by sonication in ice-cold medium containing 8 mmol/L urea and centrifuged at 12,000 g, 4°C for 15 min. Supernatants were collected and Laemmli buffer (62.5 mmol/L; Tris-HCl, pH 6.8; 25% glycerol; 2% SDS; 0.01% Bromophenol

Blue; 10 mmol/L DTT; without 2-mercaptoethanol) was added to samples. For PRDX1, PRDX3, PRDX6, p-c-Jun ser 63 and p-c-Jun ser 73, 30 μg of the total protein from the supernatants were heated at 100°C for 5 min, resolved by electrophoresis in 10% SDS-polyacrylamide gel and electro-blotted onto nitrocellulose membranes. For PRDX2, PRDX4 and PRDX5, 50 μg of protein were used instead. After blocking in 5% non-fat milk solution for 2 hours, immunodetection was performed after overnight incubation with a PRDX1 antibody (1:1000 dilution), PRDX3 antibody (1:300 dilution), PRDX6 antibody (1:1000 dilution), p-c-Jun ser 63 and ser 73 antibodies (1:1000 dilution), PI3K p85 subunit antibody (1:1000 dilution), PRDX2, PRDX4 and PRDX5 antibodies (1:500 dilution). GAPDH antibody (1:1000 dilution) was used as a loading control. Membranes were then exposed to 150 ng/mL specific secondary peroxidase-conjugated antibody (anti IgG (H+L)-HRP, Invitrogen) for 2 hours at room temperature and visualized by chemiluminescence (SuperSignal, Pierce Biotechnology Inc., Rockford, IL, USA). The bands were quantified using the Scion Image software (Scion Corp., Frederick, MD, USA).

2.4. RNA extraction and quantitative real-time PCR

RINm5F cells were collect in 1 mL of TRIzol reagent (Invitrogen). Total RNA was isolated according to the manufacturer's guidelines and quantified by a spectrophotometer. The purity and integrity of RNA was verified by agarose gel electrophoresis. Complementary DNA was prepared using 3 μg of total RNA and MultiScribe reverse transcriptase ™ (Applied Biosystems). GAPDH was used as a housekeeping gene. Real-time PCR was carried out in the StepOne thermocycler (Applied Biosystems). The PCR conditions were 95°C for 10 min, followed by 40 cycles at 95° for 10 s and 60°C for 30 s. Real-time PCR data were analysed using the Sequence Detector System 1.7 (Applied Biosystems).

2.5. MTS cell viability assay

For estimation of toxicity of IFN-gamma, TNF-alpha, H_2O_2, and of the Calpain inhibitor, the number of viable cells was determined by the colorimetric method of reduction of tetrazolium salt into soluble formazan, according to the manufacturer's instructions. The viability was expressed as % of the MTS absorbance at 490 nm in the absence of tested compound (control cells).

2.6. JNK and PRDX6 siRNA transfection

RINm5F cells were seeded on 24-well culture plates until reaching 80 % confluence. Thereafter, cells were transfected using Lipofectamine 2000 (Invitrogen) with either 100 or 200 nmol/L (final concentration) siRNA raised against JNK or against PRDX6 according to manufacturer's instructions. A time-dependent curve was performed before cell viability experiments. Sequences of the JNK siRNA (Stealth Select siRNA Duplex Oligoribonucleotides, InVitrogen, Pasley, UK) used were AAAGAATGTCCTACCTTCT (sense) and

AGAAGGTAGGACATTCTTT (anti-sense). Sequences of the PRDX6 siRNA (Silencer Select Pre-designed siRNA, Life Technologies do Brasil LTDA., São Paulo, Brazil) used were GGAAACCUCAGGUCUUGUAtt (sense) and UACAAGACCUGAGGUUUCCtc (anti-sense). Tests using a non-targeting RNA sequence (Control siRNA-A, Santa Cruz Biotechnology, Inc; catalogue number sc-37007) were made in pre-experiments, and showed that there were no changes on the results of PRDX6 levels at any of our conditions (not shown). Therefore, mock transfections were used as control thereafter. Cells were harvested for western-blotting measurements after 48, 72, and/or 96 hours of transfection, as indicated in the figures.

2.7. Statistics

All data are expressed as mean ± SEM. Statistical analyses were performed using One-way or Two-way ANOVA followed by either Bonferroni's or Dunnett's test, as required. Letters above bars denote significant differences among groups when letters are dissimilar ("a" is statistically different from "b", "c", "d", and so forth; while a group with an "a" on the top of the bar is statistically not different of another group with an "a" on the top of the bar within the same set of experiment; the same for "b" equal "b", "c" equal "c", and so forth). Only groups where differences were found were marked accordingly. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of IL-1beta, TNF-alpha and IFN-gamma on the PRDXs mRNA and protein levels in insulin-producing RINm5F cells

Exposure of insulin-producing RINm5F cells to 60 or 600 U/mL of IL-1beta, 185 or 1850 U/mL of TNF-alpha and 14 or 140 U/mL of IFN-gamma for 6 and 24 hours did not change the mRNA levels of any of the PRDX isoforms (supplementary data in Table S2). The incubation time and concentrations of the cytokines used were based on previous studies (Souza, et al., 2004; Souza et al., 2008). Likewise, incubation with these cytokines for 6 and 24 hours did not modulate the levels of PRDX1 (supplementary Fig. S1A and S1B) and PRDX3 (supplementary Fig. S1C and S1D) proteins. No changes were observed in the PRDX6 protein level after 6 hours of cytokine exposure (Fig. 1A). However, there were reductions of 49 ± 9% and 58 ± 11% in the PRDX6 levels after 24 hours of incubation with 1850 U/mL TNF-alpha and 140 U/mL IFN-gamma, respectively (Fig. 1B). Of note, we were not able to detect the PRDX2, PRDX4 or PRDX5 isoforms at the protein level in RINm5F cells, even using higher amounts of loaded protein (50 μg) (supplementary Fig. S1E). Attempts to detect PRDX2 with a distinct antibody (Epitomics, Burlingame, CA, USA, c. number 3539-1, dilution 1:1000) and high amount of loaded protein were made, but still there were no visible bands after normal western-blotting development (1 min exposition time to autoradiograms); (supplementary Fig. S2, left panel). Using very long exposition time to autoradiograms (1h) faint bands were barely detect (supplementary Fig. S2, right panel). Hence, it was clear that the amount of PRDX2 was at or below of detection limits of both the methodology and the antibody, and we can assure with good degree of certainty that RINm5F cells does not

constitutively express significant amounts of this protein. Although not explored in this work, the same might be true to PRDX4 and PRDX5.

We also confirmed the results on PRDX6 protein down-regulation in another pancreatic beta cell line, namely INS1-E, which showed the same pattern of PRDX6 protein level decrease after incubation with 1850 U/mL TNF-alpha and 140 U/mL IFN-gamma alone or in combination (supplementary Fig. S3). In addition, since no differences were found in PRDXs levels after incubation with IL-beta, opposing to previously published results (Bast, et al., 2002), we also tested whether our cells were responding to this cytokine as expected. As shown in supplementary Fig. S4, there was a marked increase in iNOS expression after incubation of RINm5F cells with 600 U/mL of IL-1beta, similar to that found after incubation of INS-1E cells, confirming the sensitivity of these cells to IL-1beta.

3.2. Effect of the combination of TNF-alpha and IFN-gamma on the PRDX6 mRNA and protein levels in insulin-producing RINm5F cells

Since there was a reduction in PRDX6 protein level after incubation with TNF-alpha or IFN-gamma, separately, we analysed RINm5F cells exposed to a combination of TNF-alpha and IFN-gamma for 24 hours. PRDX6 protein level did not change in cells incubated with TNF-alpha + IFN-gamma at the lowest concentrations (185 + 14 U/mL), but at the highest concentrations (1850 + 140 U/mL), we observed a significant decrease of 59 ± 5%, compared to control cells (Fig. 2A). PRDX6 mRNA level was not altered by TNF-alpha and IFN-gamma, separately or in combination, at the lower concentrations. However, at the highest concentration, there was a marked reduction of 65 ± 5% in the PRDX6 mRNA level (Fig. 2B). This result indicates that modulation in PRDX6 at the transcriptional level is more complex, and may require the activation of major intracellular signalling pathways. To confirm this hypothesis, insulin-producing RINm5F cells were pre-incubated with pyrrolidine dithiocarbamate (PDTC), a pharmacological inhibitor of NF-kappaB translocation into the nucleus, which is known to be a crucial pathway in pro-inflammatory cytokine signalling on beta cells (Pirot et al., 2008; Souza et al., 2008). The concentration and time of incubation of this inhibitor has been previously described (Paula, et al., 2010). After blockage of NF-kappaB translocation, no reduction in the PRDX6 mRNA level was observed in RINm5F cells incubated with TNF-alpha + IFN-gamma, indicating that the activation of NF-kappaB is necessary for decreasing PRDX6 mRNA level (Fig. 2C). Other major pathways were also involved in the modulation of PRDX6 mRNA by the combination of IFN-gamma plus TNF-alpha. The blockade of STAT1 using curcumin and of JNK pathway using SP600125 inhibitors recovers PRDX6 mRNA, albeit showing a slightly lower capacity of recovering compared to the blockade of NF-kappaB (Fig. 2C). It is important to mention here, that our results do not definitely proved that the reduction in mRNA level translates into reduced protein level or the mechanism of this decrease in mRNA level, which could be either by decreased mRNA expression or increased mRNA degradation. On the other hand, it has been previously proposed that alterations in the mRNA levels mirrors the protein levels in pancreatic islets (Cardozo, et al., 2003), corroborating the idea that mRNA levels might determine the PRDX6 protein level in the context of our work. However, proteolysis could

also be an alternative route for control of protein expression, and we then decided to test this possibility for PRDX6 modulation in RINm5F cells exposed to the diabetogenic cytokines.

3.3. Modulation of PRDX6 protein level by TNF-alpha and IFN-gamma in insulin-producing RINm5F cells requires Calpain and proteasome proteolysis systems

Viability was measured by the MTS test in order to determine the ideal concentration and time of exposure of the Calpain inhibitor. A time- and dose-dependent curve was performed, and, based on the results (Fig. 3A), the concentration chosen was 10 μmol/L. For the proteasome inhibitor (MG 132), 10 μmol/L was added to the culture medium. This concentration was selected on the basis of previously published study (Kwon, et al., 1998). There was a reduction in the PRDX6 protein level of approximately 50% when RINm5F cells were incubated with TNF-alpha and IFN-gamma for 24 hours (Fig. 3B and C). The blockage of Calpain (Fig. 3B) and proteasome (Fig. 3C) proteolysis systems completely restored the PRDX6 protein level in cells incubated with TNF-alpha and IFN-gamma alone. When both cytokines were used in combination, inhibition of these proteolytic pathways clearly (but not totally) restored the PRDX6 protein intracellular level.

3.4. Contribution of JNK signalling to the TNF-alpha- and IFN-gamma-induced decrease in PRDX6 protein level in insulin-producing RINm5F cells

We also tested the involvement of JNK signalling in the modulation of PRDX6 levels. To confirm the efficiency of the JNK inhibitor (SP600125) used in the tests, the phosphorylation of the transcription factor, c-Jun, the direct substrate of JNK, on serine 63 and serine 73 was analysed. There was a significant reduction in these two c-Jun phosphorylation sites after the blockage of JNK (Fig. 4A). Blockage of the JNK pathway abolished the reduction in the PRDX6 protein intracellular level induced by TNF-alpha and IFN-gamma (Fig. 4B). These results were confirmed using an siRNA raised against JNK1. As shown in Fig. 5, down-regulation of JNK1 also restores PRDX6 protein expression levels. It is of note here, that the recovery of PRDX6 protein quantity after blockade of JNK pathway might involve restoration of mRNA levels (as shown in Fig. 2C), which in turn might be related to c-Jun phosphorylation pattern.

3.5. Effect of IL-4 cytokine on the PRDXs mRNA levels and PRDX1, PRDX3 and PRDX6 protein levels in insulin-producing RINm5F cells

To evaluate whether the anti-inflammatory cytokine IL-4 modulates PRDXs mRNA levels, insulin-producing RINm5F cells were incubated with different concentrations of IL-4 (2, 20 and 50 ng/mL) for 6 and 24 hours. There was no effect of IL-4 alone on the mRNA levels of any of the PRDXs isoforms (supplementary data in Table S3) and on the PRDX1, PRDX3 (supplementary Figs. S3A and S3B) and PRDX6 (Fig. 6) protein levels.

3.6. Interactions between anti- and pro-inflammatory cytokines on the PRDX6 mRNA and protein levels in insulin-producing RINm5F cells

Pre-incubation with 50 ng/mL of the anti-inflammatory cytokine IL-4 for 24 hours prevented the reduction in the PRDX6 protein level induced by the incubation with TNF-alpha and IFN-gamma separately and partially prevented the effect when the combination of both pro-inflammatory cytokines was used instead (Fig. 7A). The decrease in the PRDX6 mRNA levels mediated by the exposure of RINm5F cells to the TNF-alpha and IFN-gamma mixture was completely blocked when RINm5F cells were pre-incubated with IL-4 (Fig. 7B).

3.7. Knockdown of PRDX6 by small interfering RNA (siRNA) decreases cellular resistance against both pro-inflammatory cytokines and hydrogen peroxide

PRDX6 siRNA was very effective in decreasing PRDX6 level, with maximal effectiveness at 100 nmol/L after 96 hours of incubation (Fig. 8A). Subsequent experiments were made using this PRDX6 siRNA concentration and time of exposure. Exposure of RINm5F cells to 140 U/mL of IFN-gamma or to 1850 U/mL of TNF-alpha resulted in a decrease in cellular viability of 32 ± 6% or 31 ± 6%, respectively (Fig. 8B, black bars). Exposure to both cytokines in combination resulted in an even greater decrease in cellular viability of 49 ± 4% (Fig. 8B, black bar). The knockdown of PRDX6 renders the cells much more vulnerable to the deleterious effects of both cytokines, especially to TNF-alpha, with decreases of 63 ± 4 %, 79 ± 5% and 80 ± 2% for IFN-gamma, TNF-alpha or IFN-gamma + TNF-alpha exposure, respectively (Fig. 8B, white dotted bars). The same effect is observed in cells exposed to increasing concentrations (25, 50, 100 e 200 μmol/L) of H_2O_2. Hydrogen peroxide is deleterious to RINm5F cells, as expected (Fig. 8B, black bars). Nonetheless, after knockdown of PRDX6 by siRNA cells becomes much more susceptible to the deleterious effects of H_2O_2, with a decrease of more than 50% in cell viability at 100 μmol /L of H_2O_2 (Fig. 8B, white dotted bar) compared to cells not treated with siRNA and exposed to the same concentration of H_2O_2 (Fig. 8B, black bar), and a maximum decrease in viability of 78 ± 1% at 200 μmol/L of H_2O_2 compared to control cells, i.e., cells that were not exposed to either H_2O_2 or PRDX6 siRNA (Fig. 8B, white bar), what showed that PRDX6 is functionally relevant not only in protecting cells against diabetogenic cytokines but also in protecting cells against H_2O_2, which is the standard oxidative stressor in the biological context of pancreatic beta cells and diabetes. Another interesting point that can be depicted from these results is that the extension of the decrease in viability in cells treated with the combination of cytokines is similar to the extension comprised in the interval of 100-200 μmol/L of exposure to H_2O_2 (Fig. 8B).

4. Discussion

There is scarce information regarding the effects of either anti- or pro-inflammatory cytokines on the PRDXs expression in insulin-producing cells. The peroxiredoxin system encompasses six different isoforms of enzymes with thioredoxin- or glutathione-dependent

peroxidase activity, but despite this diversity it appears that PRDXs have no redundant function (Knoops, et al., 2007; Wang, et al., 2003). In the present study, we demonstrated that half of the peroxiredoxin isoforms are expressed at considerable protein levels in the insulin-producing RINm5F cell line, namely PRDX1, PRDX3, and PRDX6. Of these, only PRDX6 is negatively modulated by pro-inflammatory cytokines. All the remaining peroxiredoxins, i.e. PRDX1 and PRDX3, are constitutively expressed following the incubation conditions used. On the other hand, PRDX2, PRDX4, and PRDX5 proteins are either expressed at low levels, not detectable by the antibodies used, or not constitutively expressed at all in RINm5F cells. Although not further explored in the present work, the low expression levels of these PRDXs might account for the susceptibility of RINm5F cells to oxidative stress. Supporting this hypothesis, it has been demonstrated recently that overexpression of PRDX4, which is endoplasmic reticulum resident protein, protects RINm5F cells against hydrogen peroxide exposure (Mehmeti, et al., 2012).

IFN-gamma and TNF-alpha activate different intracellular signalling cascades (STAT1, and NF-kappaB and/or JNK/p38, respectively). The importance of the cross-talk among the different signalling pathways for the modulation of PRDX6, especially at the mRNA level, is documented convincingly in the present work, since only in combination IFN-gamma and TNF-alpha decrease PRDX6 mRNA levels. NF-kappaB has an essential role in this process, as when NF-kappaB migration into the nucleus is blocked, the PRDX6 mRNA decrease is prevented. Corroborating our results, it has already been shown that the PRDX6 promoter region has two putative NF-kappaB sites that contribute to the regulation of PRDX6 mRNA transcription in mice lens epithelial cells (Fatma, et al., 2009). STAT1 and JNK pathway also decisively contribute to the reduction on the PRDX6 mRNA level in RINm5F cells. Indeed, after inhibition of the JNK or STAT1 pathways, the cytokine-induced decrease in PRDX6 level is blocked. Corroborating our data, other cell types exhibit some correlations between the JNK activation and PRDXs levels and activity (Han, et al., 2005; Kim, et al., 2008). Hence, other transcription factors may be necessary to regulate PRDX6 expression in addition to NF-kappaB and STAT1, such as c-Jun, which is activated by the JNK signalling pathway.

The reduction in PRDX6 protein level mediated by TFN-alpha and IFN-gamma is related to post-transcriptional proteolysis events, such as activation of the Calpains and ubiquitin-proteasome systems. Previous evidences for protein degradation by these two systems in other cell types and contexts have already been described for PRDX1 (Seo, et al., 1999), PRDX2 (Seo et al., 1999), PRDX3 (Mukhopadhyay, et al., 2006), and PRDX4 (Roumes, et al., 2010). Additionally, using an in vitro yeast two hybrid system, evidence has been found for the interaction between PRDX6 and Calpain (Budanova and Bystrova, 2008). Hence, the control of the protein expression levels by proteolysis seems to be common for the majority (if not all) of the PRDXs, and it is demonstrated here for the first time that, for PRDX6, this is a regulated pathway that can be activated by the diabetogenic pro-inflammatory cytokines TNF-alpha and IFN-gamma, and blocked by IL-4. Direct activation of Calpains have been observed in other cell types exposed to IFN-gamma (Hastie, et al., 2008; Nozaki, et al., 2010). It is also known that the Calpain proteolysis system might be important for the activation of intracellular pathways by pro-inflammatory cytokines, like activation of NF-kappaB by TNF-alpha (Han, et al., 1999), which could in turn modulate the expression of PRDX6.

IL-4 may be considered an anti-inflammatory cytokine in the context of the pancreatic beta cell, since it may protect these cells against the deleterious effects of pro-inflammatory cytokines (Kaminski et al., 2007; Kaminski et al., 2010; Souza et al., 2008). IL-4 alone does not modulate PRDX1, PRDX3 nor PRDX6 protein levels or the level of any of the PRDXs mRNAs in the insulin-producing RINm5F cells. However, IL-4 is able to counteract the reduction induced by IFN-gamma and TNF-alpha in PRDX6 mRNA and protein levels. It is known that beta cells express the IL-4 receptor and that IL-4 protects against pro-inflammatory-induced beta cell apoptosis via multiple mechanisms that may involve the activation of PI3K/Akt pathway, decreasing the activation of NF-kappaB, and decreasing the expression of iNOS, resulting in reduced caspase-3 activity and cytotoxicity (Kaminski et al., 2007; Kaminski et al., 2010; Souza et al., 2008). Of note, IL-4 may prevent PRDX6 decrease by a dual mechanism involving blockade of PRDX6 proteolysis when cells are incubated with IFN-gamma and TNF-alpha separately and additionally the restoration of PRDX6 mRNA levels when cells are incubated with both pro-inflammatory cytokines. However, it seems that IL-4 does not completely block proteolysis in the latter case, since the restoration of PRDX6 protein levels is not total when cells are incubated with both IFN-gamma and TNF-alpha in the presence of IL-4 (Fig. 7A).

Hence the events leading to beta cell death are extremely complex. The relative importance of each cytokine alone or in combination is not fully known and the relative importance of ROS and RNS is not completely established (Jorns, et al., 2005; Kaminski et al., 2007; Souza et al., 2008). This is important topic as the cytokine milieu ultimately determines both the progression of intra-islet immune cells appearance/activation and also the production of ROS and RNS by pancreatic beta cells (Jorns et al., 2005; Kaminski et al., 2007; Souza et al., 2008). A combination of oxidative and nitrosative stresses might be necessary for beta cell death in diabetes. However, evidence from animal models of diabetes indicate that IL-1beta causes dysfunction and death of beta cells, but surprisingly this seems not to be related to NO production (Todaro et al., 2003). In this complex scenario, it is conceivable that the decrease in PRDX6 induced by TNF-alpha and IFN-gamma, alone or in combination, contributes to cellular damage, probably increasing susceptibility to hydrogen peroxide, but without involvement of IL-1beta-induced NO production. Corroborating this hypothesis, it is demonstrated in this work that PRDX6 has a functional role in the insulin-producing RINm5F cells, which is relevant for protection of cells against oxidative stress. We also know, from preliminary results, that PRDX6 down-regulation might activate caspase-3 in pancreatic beta cell lineages, which is further evidence of the importance of this peroxiredoxin for beta cell death (not shown). The relevance and final validation of this hypothesis to the diabetes development will have however to be complemented with isolated islets from future in vivo experiments. Use of PRDX6 knockdown mice seems a promising strategy.

In summary, our results show that incubation of insulin-producing RINm5F cells with pro-inflammatory cytokines, namely IFN-gamma and TNF-alpha, leads to the down-regulation of PRDX6. No other peroxiredoxin isoforms are modulated by these cytokines. The regulation of PRDX6 intracellular levels encompasses two distinct events, proteolysis at the protein level and control of gene expression at the mRNA level. Only when used in combination do IFN-gamma and TNF-alpha decrease PRDX6 mRNA level, implying the need for a concomitant activation of different transcription factors and possibly other accessory proteins. Separately, IFN-gamma and

TNF-alpha are able to reduce PRDX6 protein level but not mRNA level. There is a direct involvement of Calpain proteases and the ubiquitin-proteasome system in this proteolytic process. NF-kappaB is involved in these events, at least when TNF-alpha signals into beta cells. Alone, the anti-inflammatory cytokine IL-4 does not modulate any of the PRDXs isoforms. However, IL-4 is able to counteract the reduction in the PRDX6 mRNA and protein levels induced by IFN-gamma and TNF-alpha. The molecular pathways involved in the PRDX6 modulation by cytokines are outlined in Fig. 9. These results indicate that PRDX6 is important for protection of beta cells against oxidative stress and strengthen our knowledge regarding the relationship between anti- and pro-inflammatory intracellular signalling cascades in insulin-producing cells.

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Figures and Legends

Fig. 1

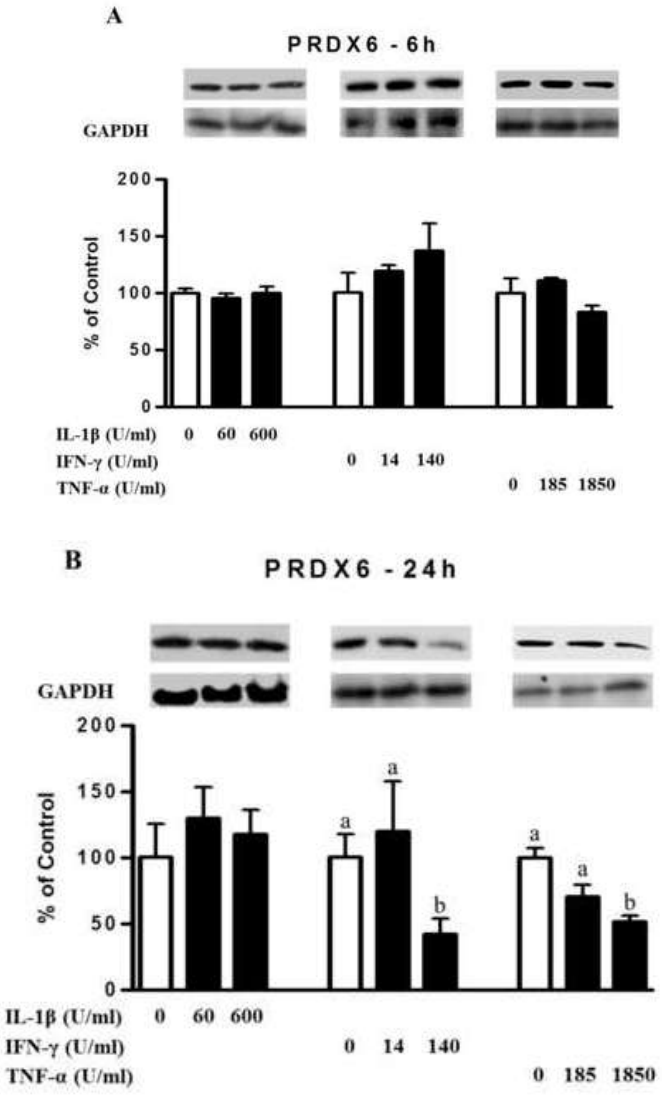
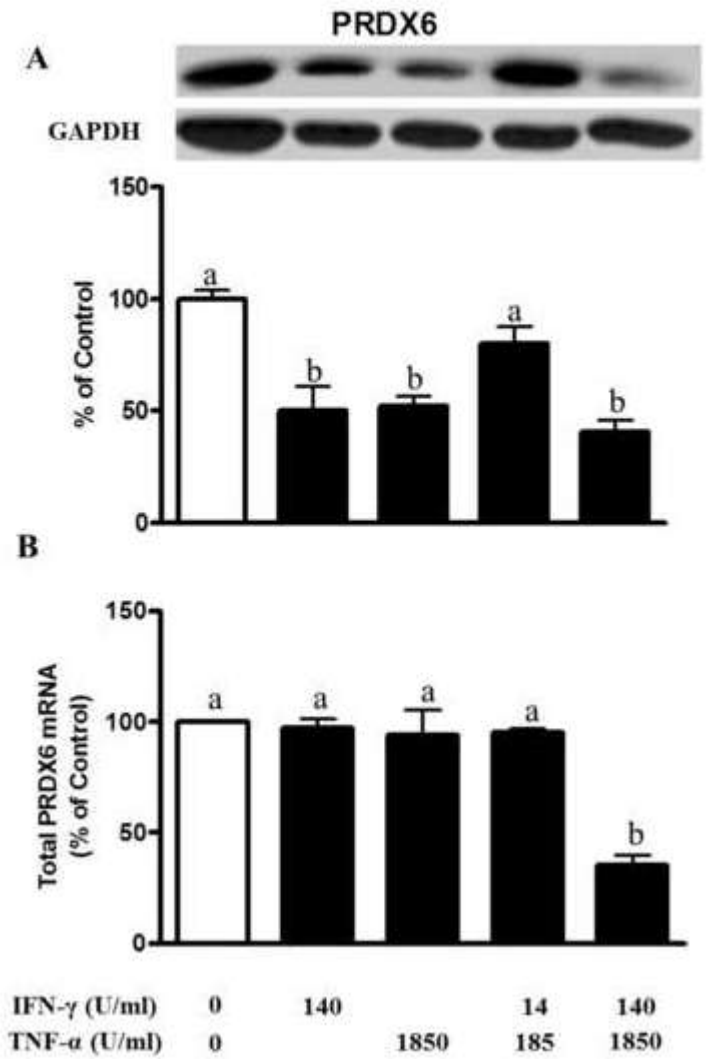


Fig. 1. *Effect of IL1-β, IFN-γ and TNF-α upon the PRDX6 protein level in insulin-producing RINm5F cells.* RINm5F cells were seeded 24 hours before incubation with 60 or 600 U/mL of IL1-β, 14 or 140 U/mL of IFN-γ and 185 or 1850 U/mL of TNF-α, for 6 and 24 hours. After this period, cells were lysed and used for the Western-Blotting analyses. (A) PRDX6 protein level after 6 and (B) 24 hours of incubation. Blots on the top are representative of 4 independent experiments normalised to the housekeeping protein GAPDH. Results are presented as mean ± S.E.M., compared against control values obtained from cells incubated without cytokines. Different letters indicate statistically significant differences. Every 3 groups comprise a set of experiment and was statistically analysed separated from the others, and marked accordingly with the letters. When no differences were found, no letter was assigned to the group. $P < 0.05$; One-way ANOVA followed by Bonferroni.

Fig. 2


A
PRDX6
GAPDH
150
100
50
0
% of Control
a
b
b
a
b
B
Total PRDX6 mRNA (% of Control)
150
100
50
0
a
a
a
a
b
IFN-γ (U/ml) 0 140 14 140
TNF-α (U/ml) 0 1850 185 1850

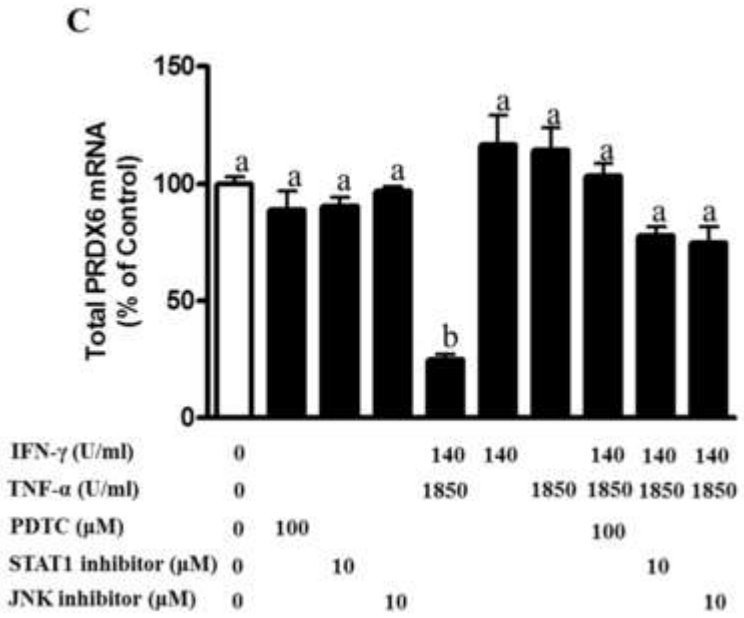
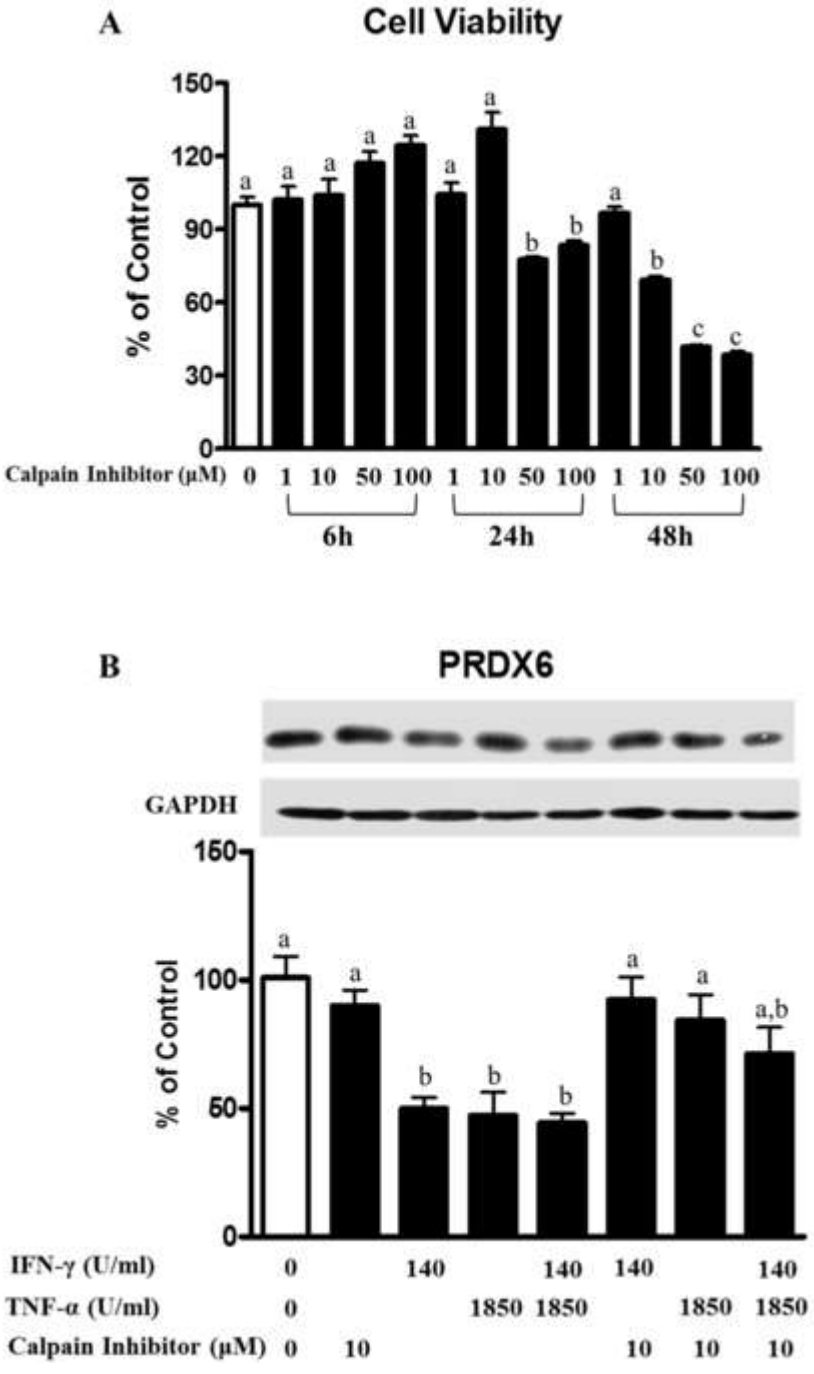


Fig. 2. *Effect of IFN-γ + TNF-α cytokines on the PRDX6 protein and mRNA levels in insulin-producing RINm5F cells.* RINm5F cells were seeded 24 hours before incubation with IFN-γ and TNF-α alone or in a combination for 24 hours. After this period, cells were lysed and used for Western-Blotting or real-time PCR analyses. (A) The PRDX6 protein and (B) mRNA levels were analyzed after exposure of cells with cytokines. (C) The PRDX6 mRNA was analysed after cells were exposed to cytokines in the presence or absence of PDTC, STAT1 inhibitor or JNK inhibitor. Different letters indicate statistically significant differences among each treatment. $P < 0.001$. Data are expressed as mean ± S.E.M., normalised to the housekeeping GAPDH and represent 4 independent. Two-way ANOVA followed by Bonferroni.

Fig. 3


A
Cell Viability
% of Control
150
120
90
60
30
0
a a a a a a b b a b c c
Calpain Inhibitor (μM) 0 1 10 50 100 1 10 50 100 1 10 50 100
6h
24h
48h
B
PRDX6
GAPDH
% of Control
150
100
50
0
a a b b b a a a,b
IFN-γ (U/ml) 0 140 140 140 140
TNF-α (U/ml) 0 1850 1850 1850 1850
Calpain Inhibitor (μM) 0 10 10 10 10

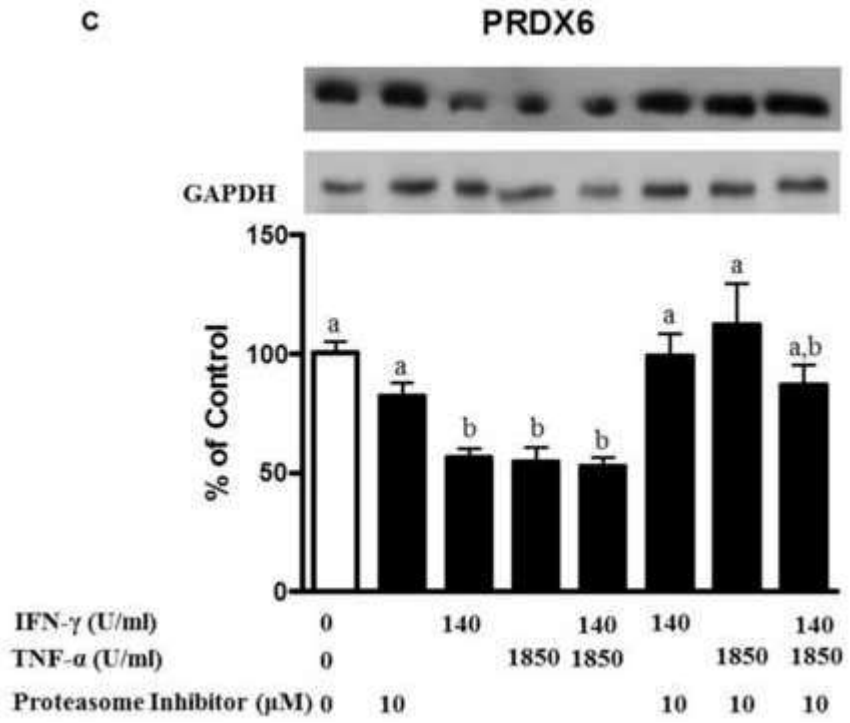


Fig. 3. *Blockade of proteolysis counteracted the cytokines-induced PRDX6 down-regulation in insulin-producing RINm5F cells.* (A) Cell viability after exposure of insulin-producing RINm5F cells to increasing concentrations of the Calpain inhibitor. RINm5F cells were seeded 24 hours before exposure to 1, 10, 50 and 100 μmol/L of Calpain inhibitor for 6, 24 and 48 hours. Control cells are grown in the absence of the inhibitor. MTS conversion to a soluble formazan product by viable cells was determined by a kinetic measurement of absorbance at 490 nm. N=7. Different letters indicate statistically significant differences. $P < 0.01$. One-way ANOVA followed by Dunnett. (B) Pre-incubation with the Calpain inhibitor blocks the reduction on the PRDX6 protein level induced by IFN-γ and TNF-α in insulin-producing RINm5F cells. RINm5F cells were seeded 24 hours before incubation with IFN-γ and TNF-α for 24 hours in the presence or absence of 10 μmol/L Calpain inhibitor. (C) Effect of blockage of proteasome system on the PRDX6 protein level in insulin-producing RINm5F cells exposed to IFN-γ and TNF-α. RINm5F cells were seeded 24 hours before incubation with IFN-γ and TNF-α for 24 hours in the presence or absence of 10 μmol/L specific proteasome inhibitor, MG132. After incubation period, cells in (B) and (C) were lysed and used for Western-Blotting analyses. Blots represent 4 independent experiments, normalised to the housekeeping protein GAPDH. Results are presented as mean ± S.E.M. Different letters indicate statistically significant differences. $P < 0.001$. Two-way ANOVA followed by Bonferroni.

Fig. 4

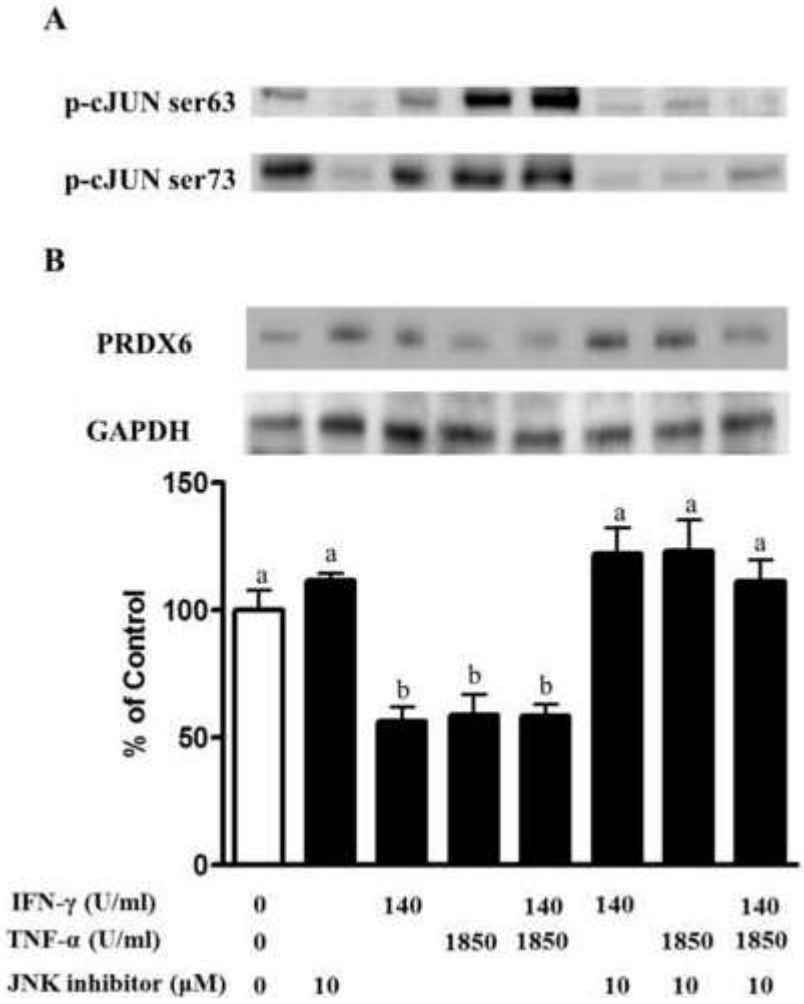


Fig. 4. *Effect of JNK inhibition on the IFN-γ and TNF-α-induced reduction on the PRDX6 protein level in insulin-producing RINm5F cells.* RINm5F cells were seeded 24 hours before incubation with IFN-γ and TNF-α for 24 hours in the presence or absence of 10 μmol/L of the JNK inhibitor SP600125. After this period, cells were lysed and used for Western-Blotting analyses. Blots on the top are representative of 4 independent experiments, normalised to the housekeeping protein GAPDH. (A) JNK-dependent phosphorylation of the transcription factor c-Jun on ser63 and ser73 after exposure to IFN-gamma and TNF-alpha. (B) Effect of blockage of JNK signalling pathway on the PRDX6 protein level in insulin-producing RINm5F cells exposed to IFN-γ and TNF-α. Results are presented as mean ± S.E.M. Different letters indicate statistically significant differences. $P < 0.001$. Two-way ANOVA followed by Bonferroni.

Fig. 5

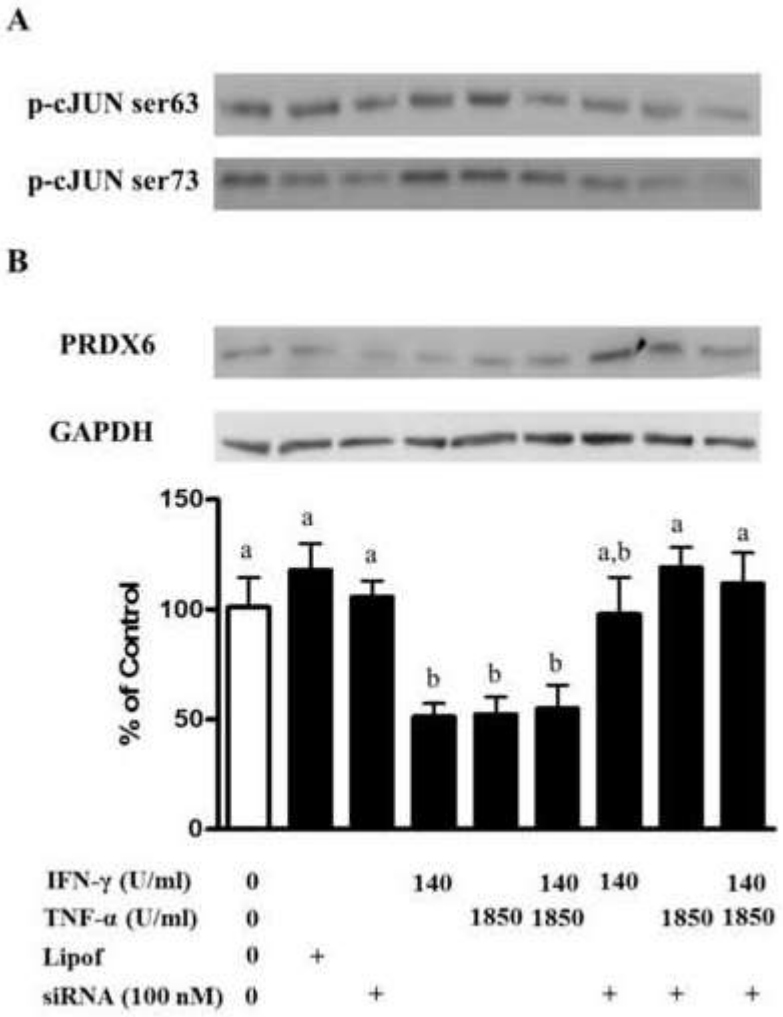


Fig. 5. *Effect of JNK inhibition by siRNA on the IFN-γ and TNF-α-induced reduction on the PRDX6 intracellular protein level in insulin-producing RINm5F cells.* RINm5F cells were incubated with 100 nmol/L siRNA against JNK1 for 48h. After this period, RINm5F cells were exposed to IFN-γ and TNF-α for additional 24 hours. Cells were lysed and used for Western-Blotting analyses. Blots on the top are representative of 4 independent experiments, normalised to the housekeeping protein GAPDH. (A) JNK-dependent phosphorylation of the transcription factor c-Jun on ser63 and ser73 after exposure to IFN-gamma and TNF-alpha. (B) Effect of the knocking down of JNK1 on the PRDX6 protein level in insulin-producing RINm5F cells exposed to IFN-gamma and TNF-alpha. Results are presented as mean ± S.E.M. Different letters indicate statistically significant differences. $P < 0.05$. Two-way ANOVA followed by Bonferroni.

Fig. 6

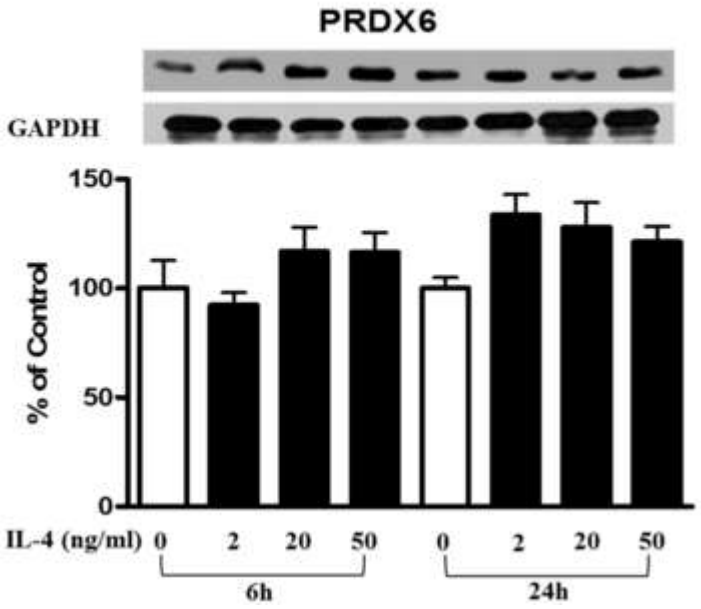


Fig. 6. *Effect of IL-4 on the PRDX6 protein level in insulin-producing RINm5F cells.* RINm5F cells were seeded 24 hours before incubation with 2, 20 and 50 ng/mL of IL-4 for 6 and 24 hours. After this period, cells were lysed and used for the Western-Blotting analyses. Blots on the top are representative of 6 independent experiments normalised to the housekeeping protein GAPDH. Results are presented as mean ± S.E.M., compared against respective control values obtained from cells incubated without cytokine. No differences were found. One-way ANOVA followed by Bonferroni.

Fig. 7

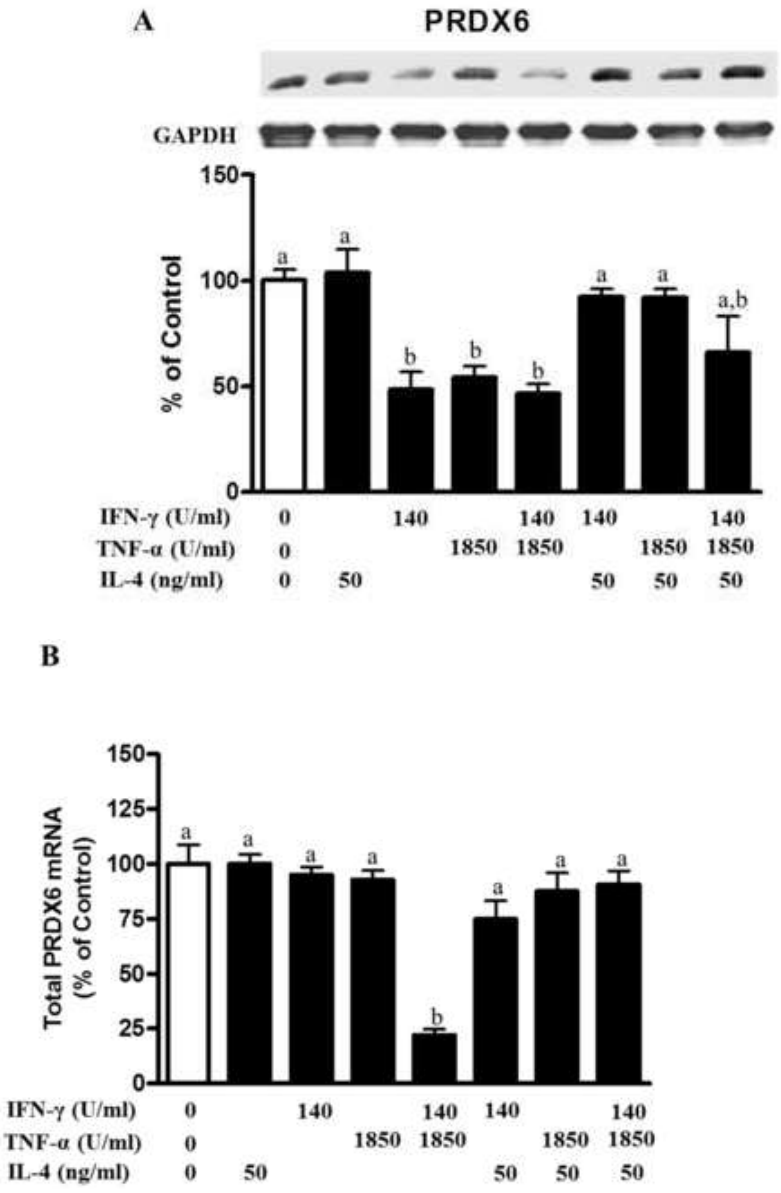


Fig. 7. *Pre-incubation with IL-4 blocks the reduction on the PRDX6 protein and mRNA levels induced by IFN-γ and TNF-α in insulin-producing RINm5F cells.* RINm5F cells were seeded 24 hours before pre-incubation with 50 ng/mL of IL-4 for 24 hours and after incubation with IFN-γ and TNF-α for additional 24 hours. After this period, cells were lysed and used for Western-Blotting or real-time PCR analyses. The (A) PRDX6 protein and (B) mRNA levels were analyzed after exposure of cells with cytokines. Different letters indicate statistically significant differences. $P < 0.001$. Data are expressed as mean ± S.E.M., normalised to the housekeeping GAPDH and represent 4 independent. Two-way ANOVA followed by Bonferroni.

Fig. 8

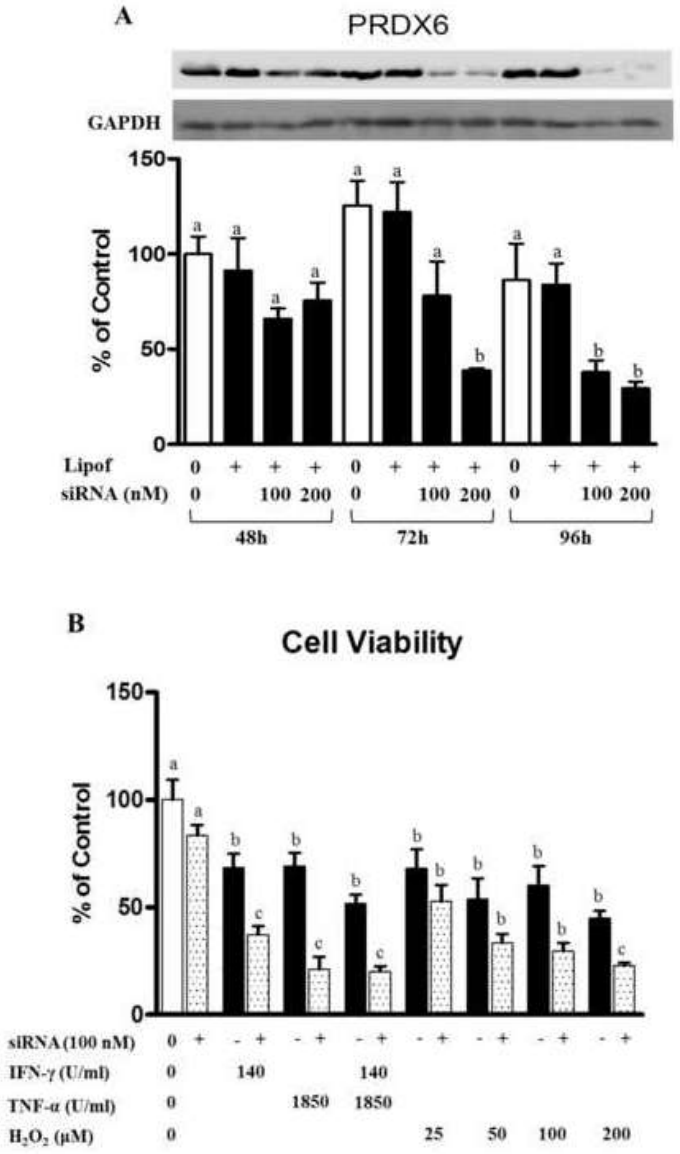


Fig. 8. *Importance of PRDX6 to cellular viability after exposure of insulin-producing RINm5F cells to TNF-α, IFN-γ or H_2O_2.* Cells were seeded 24 hours before incubation with the compounds. (A) 100 or 200 nmol/L of siRNA against PRDX6 for 48, 72 or 96 hours. After this period, cells were lysed and used for Western-Blotting. Blots on the top are representative of 4 independent experiments, normalised to the housekeeping protein GAPDH. (B) Viability of RINm5F cells exposed to IFN-γ, TNF-α or increasing concentrations (25, 50, 100 e 200 μmol/L) of H_2O_2 after knockdown of PRDX6 by means of treatment with PRDX6 siRNA for 96h. MTS conversion to a soluble formazan product by viable cells was determined kinetically at 490 nm. N=6. Results are presented as mean ± S.E.M. Different letters indicate statistically significant differences. $P < 0.001$. Two-way ANOVA followed by Bonferroni.

Fig. 9

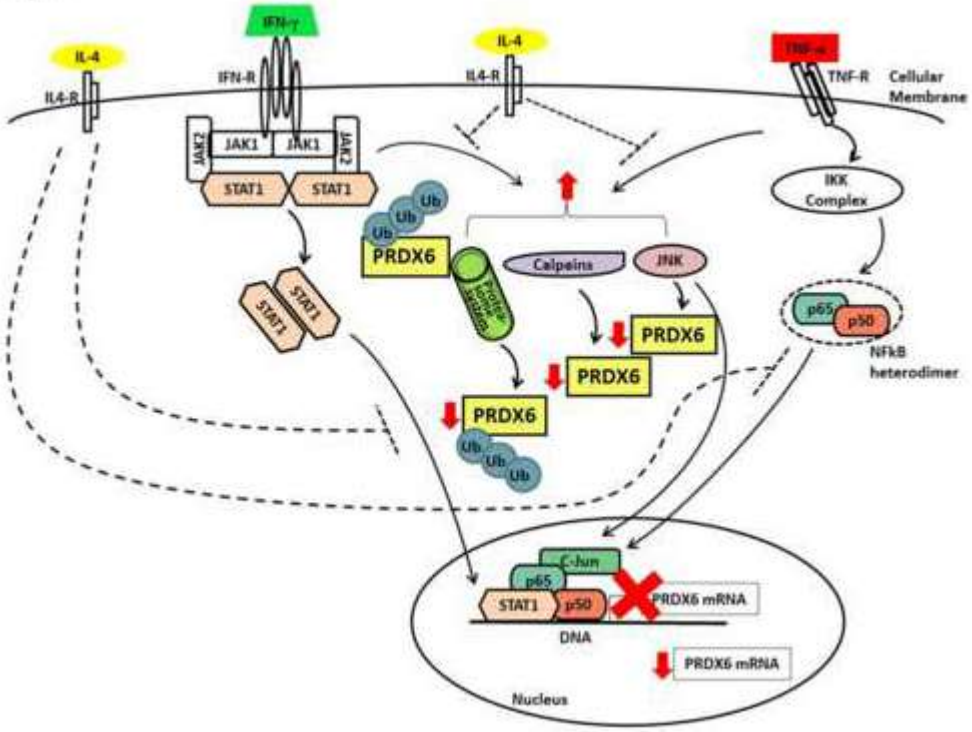


Fig. 9. *PRDX6 mRNA and protein levels and related intracellular pathways after exposure of RINm5F cells to anti- and pro-inflammatory cytokines.* IFN-gamma- and TNF-alpha-dependent down-regulation of PRDX6 protein level occurs through activation of proteolysis systems, such as calpains and proteasome, and activation of the JNK pathway. When these cytokines act in combination, they also decrease PRDX6 mRNA level, an effect that is dependent on the activation of major intracellular signalling pathways, namely STAT1 and NF-kappaB activated by IFN-gamma and TNF-alpha, respectively, and possibly JNK/c-Jun pathway modulated by both pro-inflammatory cytokines. It is not yet known whether down-regulation of PRDX6 mRNA by these transcription factors occurs directly by trans-repression or indirectly via expression of other regulatory mRNA and proteins. The anti-inflammatory cytokine IL-4 is able to counteract the IFN-gamma- and TNF-alpha-induced down-regulation of PRDX6, partially preventing PRDX6 proteolysis and totally recovering PRDX6 mRNA intracellular levels.

Table S1. Sequence of primers used for the quantification of Real Time PCR gene expression.

Gene	GenBank Access	Sequence
PRDX1	NM_057114	Fw 5'-CATGACGTGGTGTGATTCC-3'
		Rv 5'-GACTGATATAAGATGGTCTGCCCC-3'
PRDX2	NM_017169	Fw 5'-TCTAAAGGGGCTGGAGGCT-3'
		Rv 5'-TCAGGGCAGGGTCACTATTC-3'
PRDX3	NM_022540	Fw 5'-AAGGCGTTCCAGTTTGTG-3'
		Rv 5'-GCTGTTGGACTTGGCTTGAT-3'
PRDX4	NM_053512	Fw 5'-AGGGCTTGGAGAGTGATGAT-3'
		Rv 5'-TCTTGGCTTTGCTTAGGTGC-3'
PRDX5	NM_053610	Fw 5'-CAAGGGCGTAGTAAAGGCAC-3'
		Rv 5'-AGGAGATGGGAGAGTCAGAGG-3'
PRDX6	NM_053576	Fw 5'-TGGAAGAAGGGAGAGAGTGTG-3'
		Rv 5'-AGACTTAAGGCTGGGGCGTAT-3'
GAPDH	NM_017008	Fw 5'-GGAGAAACCTGCCAAGTATGATG-3'
		Rv 5'-AACCTGGTCCTCAGTGTAGCCC-3'

Fw: forward sequence (sense); Rv: reverse sequence (antisense). All amplicons were in the size ranging from 75-138 base pairs.

Table S2. Real Time PCR gene expression quantification of PRDXs gene after exposure of insulin-producing RINm5F cells to IL-1beta, TNF-alpha or IFN-gamma cytokines.

Group	PRDX1	PRDX2	PRDX3	PRDX4	PRDX5	PRDX6
Control 6h	1.00 ± 0.13	1.00 ± 0.11	1.00 ± 0.08	1.00 ± 0.09	1.00 ± 0.02	1.00 ± 0.14
60U IL-1β 6h	0.95 ± 0.22	1.19 ± 0.31	0.92 ± 0.11	0.95 ± 0.14	1.02 ± 0.21	1.06 ± 0.07
600U IL-1β 6h	0.93 ± 0.22	1.12 ± 0.31	0.94 ± 0.06	0.96 ± 0.19	1.19 ± 0.40	0.93 ± 0.21
185U TNF-α 6h	0.99 ± 0.18	1.05 ± 0.06	0.94 ± 0.05	1.07 ± 0.11	1.01 ± 0.12	1.02 ± 0.28
1850U TNF-α 6h	0.86 ± 0.17	0.96 ± 0.14	0.86 ± 0.05	0.96 ± 0.06	0.94 ± 0.02	0.88 ± 0.26
14U IFN-γ 6h	1.02 ± 0.53	0.74 ± 0.23	1.11 ± 0.42	0.98 ± 0.15	1.13 ± 0.22	0.95 ± 0.09
140U IFN-γ 6h	1.25 ± 0.59	0.98 ± 0.11	1.23 ± 0.47	1.12 ± 0.11	1.22 ± 0.26	1.07 ± 0.13
Control 24h	1.00 ± 0.13	1.01 ± 0.08	1.00 ± 0.13	1.00 ± 0.09	1.00 ± 0.08	1.00 ± 0.12
60U IL-1β 24h	0.87 ± 0.11	1.09 ± 0.28	1.03 ± 0.24	0.98 ± 0.37	0.96 ± 0.24	0.83 ± 0.01
600U IL-1β 24h	0.90 ± 0.20	0.98 ± 0.20	0.88 ± 0.16	1.02 ± 0.48	1.03 ± 0.30	0.86 ± 0.02
185U TNF-α 24h	1.07 ± 0.16	0.99 ± 0.17	0.97 ± 0.15	0.96 ± 0.10	1.06 ± 0.15	0.96 ± 0.28
1850U TNF-α 24h	1.02 ± 0.08	1.03 ± 0.05	0.95 ± 0.03	0.90 ± 0.07	0.97 ± 0.06	0.93 ± 0.23
14U IFN-γ 24h	1.15 ± 0.47	1.02 ± 0.17	1.10 ± 0.33	1.00 ± 0.14	1.20 ± 0.24	0.87 ± 0.22
140U IFN-γ 24h	1.20 ± 0.48	1.11 ± 0.23	0.92 ± 0.20	0.98 ± 0.26	1.23 ± 0.23	0.97 ± 0.08

Insulin-producing RINm5F cells were seeded 24 hours before incubation with two different concentrations of pro-inflammatory cytokines, IL-1beta, TNF-alpha or IFN-gamma, for 6 and 24 hours. After these periods, the cells were lysed, mRNA was isolated and used for the Real Time PCR analyses. Results are presented as -fold variation ± S.E.M. and compared against control values obtained from cells incubated without cytokines, and represent 4 independent experiments, normalised to the housekeeping gene GAPDH. One-way ANOVA followed by Bonferroni. IL-1β: Interleukin 1 beta; TNF-α: Tumour Necrosis Factor alpha; IFN-γ: Interferon gamma.

Table S3. Real Time PCR gene expression quantification of PRDXs gene after exposure of insulin-producing RINm5F cells to anti-inflammatory cytokine IL-4.

Group	PRDX1	PRDX2	PRDX3	PRDX4	PRDX5	PRDX6
Control 6h	1.00 ± 0.05	1.00 ± 0.22	1.00 ± 0.13	1.00 ± 0.10	1.00 ± 0.10	1.00 ± 0.10
2 ng IL-4 6h	1.00 ± 0.05	0.88 ± 0.19	0.97 ± 0.14	1.15 ± 0.21	0.95 ± 0.14	0.94 ± 0.04
20 ng IL-4 6h	0.96 ± 0.13	1.05 ± 0.10	0.97 ± 0.10	1.32 ± 0.2	1.04 ± 0.15	0.93 ± 0.05
50 ng IL-4 6h	1.04 ± 0.17	0.94 ± 0.05	0.99 ± 0.01	1.27 ± 0.1	0.83 ± 0.13	0.98 ± 0.10
Control 24h	1.00 ± 0.10	1.00 ± 0.18	1.00 ± 0.10	1.00 ± 0.10	1.00 ± 0.13	1.00 ± 0.10
2 ng IL-4 24h	0.98 ± 0.10	1.12 ± 0.21	1.03 ± 0.12	1.08 ± 0.13	0.96 ± 0.28	0.86 ± 0.12
20 ng IL-4 24h	0.95 ± 0.13	1.15 ± 0.30	1.00 ± 0.23	1.09 ± 0.15	1.05 ± 0.32	0.91 ± 0.19
50 ng IL-4 24h	0.97 ± 0.15	1.03 ± 0.13	1.09 ± 0.26	0.99 ± 0.15	0.98 ± 0.11	0.85 ± 0.10

Insulin-producing RINm5F cells were seeded 24 hours before incubation with different concentrations of anti-inflammatory cytokine IL-4 for 6 and 24 hours. After these periods, the cells were lysed, mRNA was isolated and used for the Real Time PCR analyses. Results are presented as -fold variation ± S.E.M. and compared against control values obtained from cells incubated without IL-4, and represent 4 independent experiments, normalised to the housekeeping gene GAPDH. One-way ANOVA followed by Bonferroni. IL-4: Interleukin 4.

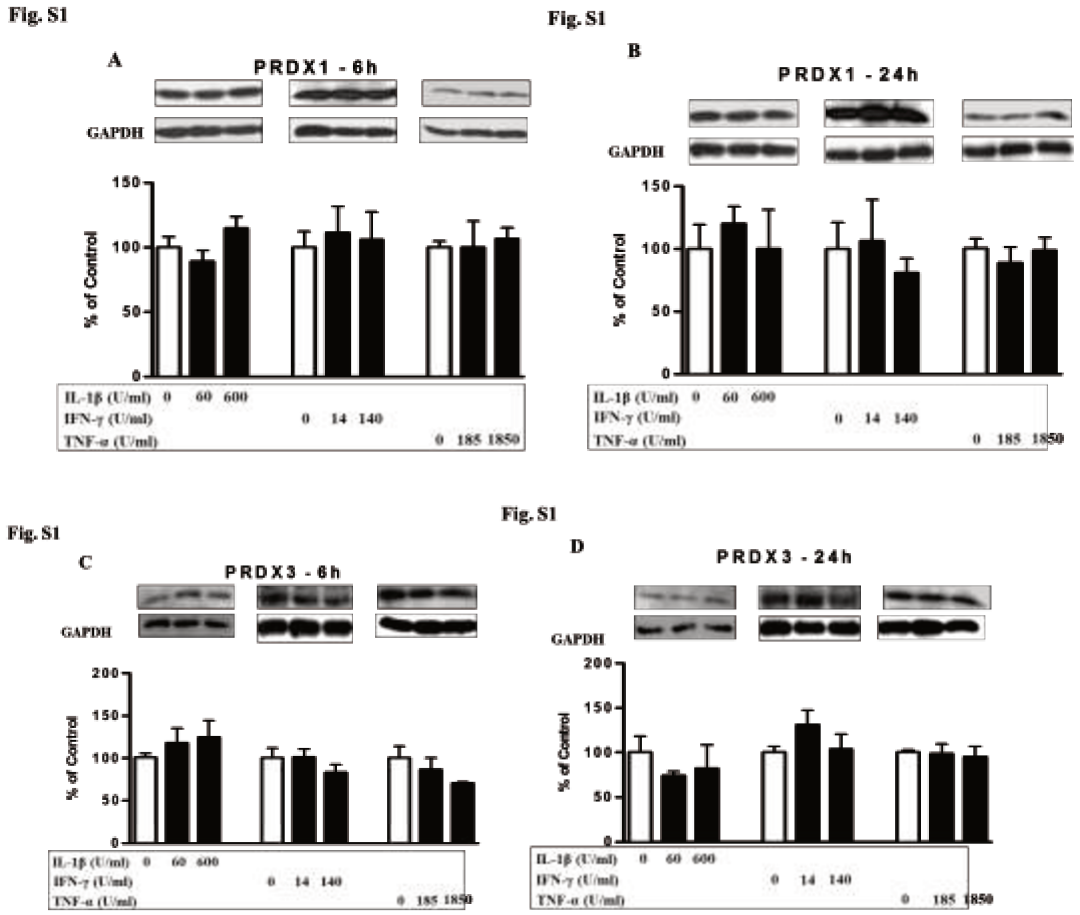


Fig. S1. *Effect of IL1-β, IFN-γ and TNF-α upon the PRDX1, PRDX2, PRDX3, PRDX4 and PRDX5 proteins expression in insulin-producing RINm5F cells.* RINm5F cells were seeded 24 hours before incubation with 60 or 600 U/mL of IL1-β, 14 or 140 U/mL of IFN-γ and 185 or 1850 U/mL of TNF-α, for 6 and 24 hours. After this period, cells were lysed and used for the Western-Blotting analyses. (A) PRDX1 protein expression after 6 and (B) 24 hours of incubation. (C) PRDX3 protein expression after 6 and (D) 24 hours of incubation. (E) The absence of protein expression of PRDX2, PRDX4 and PRDX5 in RINm5F cells. Blots on the top are representative of 4 independent experiments normalised to the housekeeping protein GAPDH. Results are presented as mean ± S.E.M., compared against control values obtained from cells incubated without cytokines.

Fig. S2

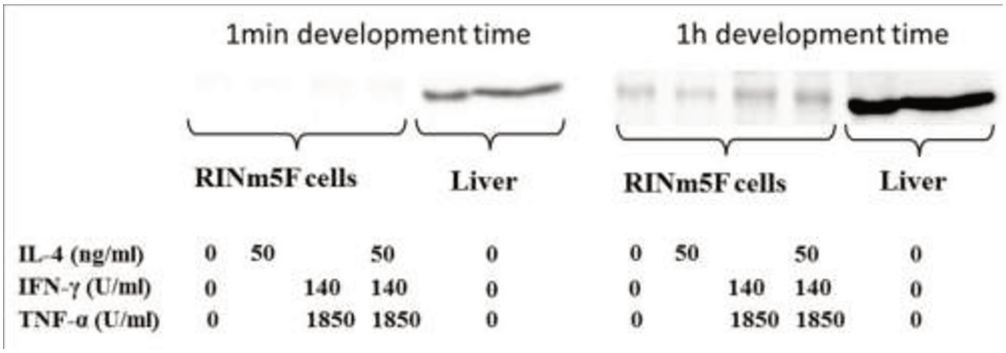


Fig. S2. *Confirmation of absence of PRDX2 protein expression in RINm5F cells using a diverse antibody.* RINm5F cells were seeded 24 hours before incubation with indicated cytokines for further 24h. The control group was cultivated in normal RPMI 1640 medium without addition of cytokines. After this period, cells were lysed and used for the Western-Blotting analyses. RINm5F samples were loaded with 100 mg of protein. The first smaller band of the liver group was from 50 mg of loaded protein and the second broader band of the liver group was from 100 mg of protein. Blots are representative of 2 independent experiments.

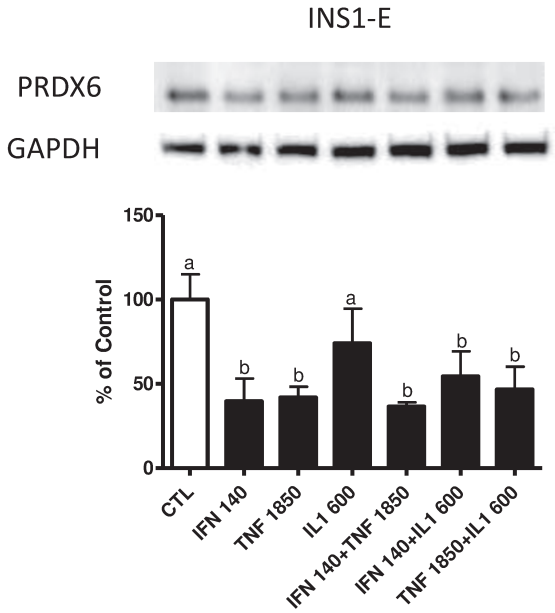


Fig. S3. *Effect of IL1-β, IFN-γ and TNF-α upon the PRDX6 protein level in insulin-secreting INS1-E cells.* INS1-E cells were seeded 24 hours before incubation with 600 U/mL of IL1-β, 140 U/mL of IFN-γ, 1850 U/mL of TNF-α and combinations of these cytokines (as indicated in the figure), for further 24 hours. After this period, cells were lysed and used for the Western-Blotting analyses. Blots on the top are representative of 4 independent experiments normalised to the housekeeping protein GAPDH. Results are presented as mean ± S.E.M., compared against control values obtained from cells incubated without cytokines. Different letters indicate statistically significant differences. $P < 0.05$; One-way ANOVA followed by Bonferroni.

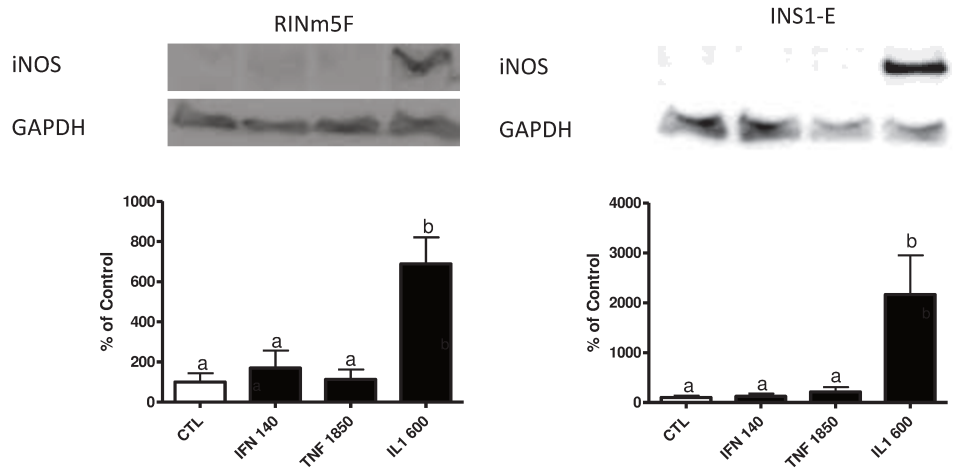


Fig. S4. *Confirmation of sensitivity of RINm5F and INS-1E cells to IL-1beta.* Cells were seeded 24 hours before incubation with 140 U/mL of IFN-γ, 1850 U/mL of TNF-α, or 600 U/mL of IL1-β, for 24 hours. The control group was cultivated in normal RPMI 1640 medium without addition of cytokines. After this period, cells were lysed and used for the Western-Blotting analyses. There was a marked increase in iNOS expression following IL-1beta incubation. This result confirms the sensitivity of both pancreatic cell lineages to this cytokine. Blots on the top of the figures are representative of 4 independent experiments normalised to the housekeeping protein GAPDH. The results are presented as Mean ± SEM. Different letters indicate statistically significant differences ($P < 0.05$, One-way ANOVA followed by Bonferroni).

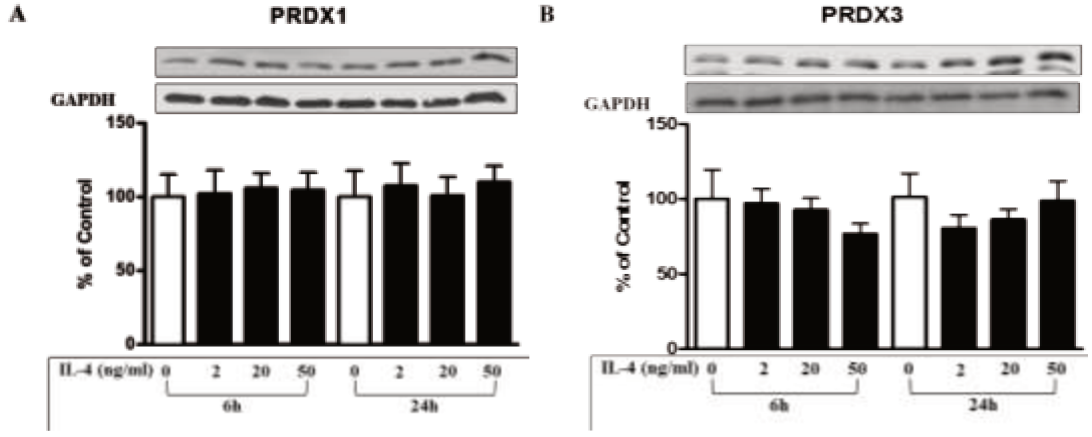


Fig. S5. *Effect of IL-4 on the PRDX1 and PRDX3 protein expression in insulin-producing RINm5F cells.* RINm5F cells were seeded 24 hours before incubation with 2, 20 and 50 ng/mL of IL-4 for 6 and 24 hours. After this period, cells were lysed and used for the Western-Blotting analyses. PRDX1 (A) and PRDX3 (B) protein expression after exposure the cells with IL-4. Blots on the top are representative of 6 independent experiments normalised to the housekeeping protein GAPDH. Results are presented as mean ± S.E.M., compared against respective control values obtained from cells incubated without cytokine.

CONCLUSÕES

Conclusões

Nossos resultados permitem concluir que:

a) As citocinas pró-inflamatórias IFN-gamma e TNF-alpha reduziram a expressão gênica e protéica de PRDX6 em células beta produtoras de insulina RINm5F (Figura 9).

b) A diminuição na expressão protéica de PRDX6 ocorreu pela ativação de sistemas de proteólise, como: ativação de calpaínas e sistema ubiquitina-proteassomo, e ativação da via de sinalização JNK (Figura 9).

c) Somente quando associadas, IFN-gamma e TNF-alpha diminuíram a expressão gênica de PRDX6, sugerindo a necessidade de ativação concomitante dos fatores de transcrição, STAT1, NFkB, para modulação gênica de PRDX6 (Figura 9).

d) A incubação com a citocina anti-inflamatória IL-4 não aumentou a expressão de nenhuma das isoforma de PRDXs, no entanto, a pré-incubação com IL-4 seguida de exposição ao IFN-gamma e TNF-alpha preveniu a ação inibitória destas citocinas tanto sobre a expressão gênica quanto protéica de PRDX6 (Figura 9).

e) Quando diminuída, a PRDX6 aumenta a suceptibilidade das células beta produtoras de insulina RINm5F aos efeitos deletérios de IFN-gamma, TNF-alpha e H_2O_2, sugerindo o efeito protetor dessa enzima ao estresse oxidativo.

Nossos resultados reforçam o conhecimento sobre a sinalização desencadeada por citocinas anti- e pró-inflamatórias na modulação da expressão de PRDXs em linhagem de células beta produtoras de insulina RINm5F, bem como a função da PRDX6 na proteção dessas células aos efeitos danosos das citocinas pró-inflamatórias e H_2O_2.

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APÊNDICE 1

Apêndice 1

Durante 1 ano do seu doutorado (janeiro/2012 à dezembro/2012), a aluna realizou estágio no exterior, no Laboratório de Medicina Experimental, Bruxelas, Bélgica, sob supervisão do Dr. Décio L. Eizirik, em que participou do desenvolvimento do artigo "GLIS3, a Susceptibility Gene for Type 1 and Type 2 Diabetes, Modulates Pancreatic Beta Cell Apoptosis Via Regulation of a Splice Variant of the BH3-Only Protein Bim" (resumo abaixo), publicado na revista Plos Genetics.

GLIS3, a Susceptibility Gene for Type 1 and Type 2 Diabetes, Modulates Pancreatic Beta Cell Apoptosis Via Regulation of a Splice Variant of the BH3-Only Protein Bim.

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Mutations in human Gli-similar (GLIS) 3 protein cause neonatal diabetes. The GLIS3 gene region has also been identified as a susceptibility risk locus for both type 1 and type 2 diabetes. GLIS3 plays a role in the generation of pancreatic beta cells and in insulin gene expression, but there is no information on the role of this gene on beta cell viability and/or susceptibility to immune- and metabolic-induced stress. GLIS3 knockdown (KD) in INS-1E cells, primary FACS-purified rat beta cells and human islet cells decreased expression

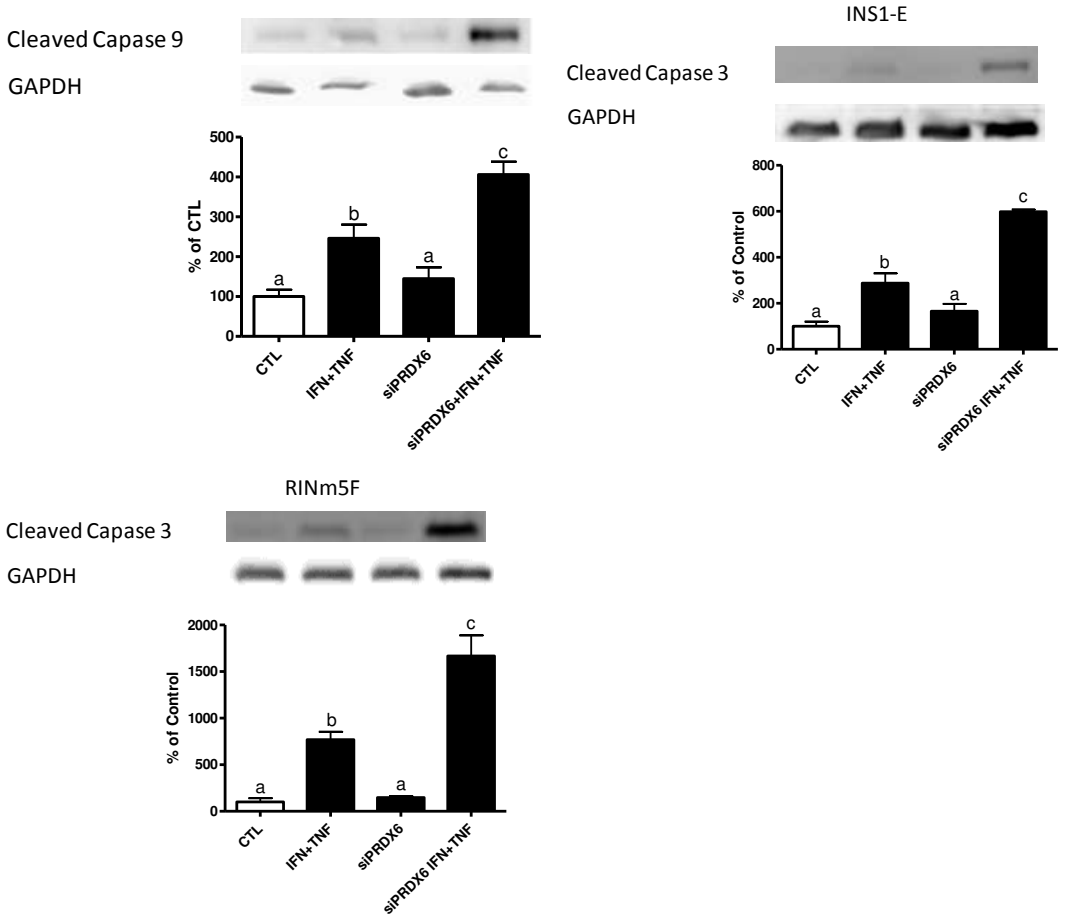
of MafA, Ins2 and Glut2 and inhibited glucose oxidation and insulin secretion, confirming the role of this transcription factor for the beta cell differentiated phenotype. GLIS3 KD increased beta cell apoptosis basally and sensitized the cells to death induced by pro-inflammatory cytokines (interleukin 1β + interferon-γ) or palmitate, agents that may contribute to beta cell loss in respectively type 1 and 2 diabetes. The increased cell death was due to activation of the intrinsic (mitochondrial) pathway of apoptosis, as indicated by cytochrome c release to the cytosol, Bax translocation to the mitochondria and activation of caspases 9 and 3. Analysis of the pathways implicated in beta cell apoptosis following GLIS3 KD indicated modulation of alternative splicing of the pro-apoptotic BH3-only protein Bim, favouring expression of the pro-death variant Bim_S via inhibition of the splicing factor SRp55. KD of Bim abrogated the pro-apoptotic effect of GLIS3 loss of function alone or in combination with cytokines or palmitate. The present data suggest that altered expression of the candidate gene GLIS3 may contribute to both type 1 and 2 type diabetes by favouring beta cell apoptosis. This is mediated by alternative splicing of the pro-apoptotic protein Bim and exacerbated formation of the most pro-apoptotic variant Bim_S.

APÊNDICE 2

Apêndice 2

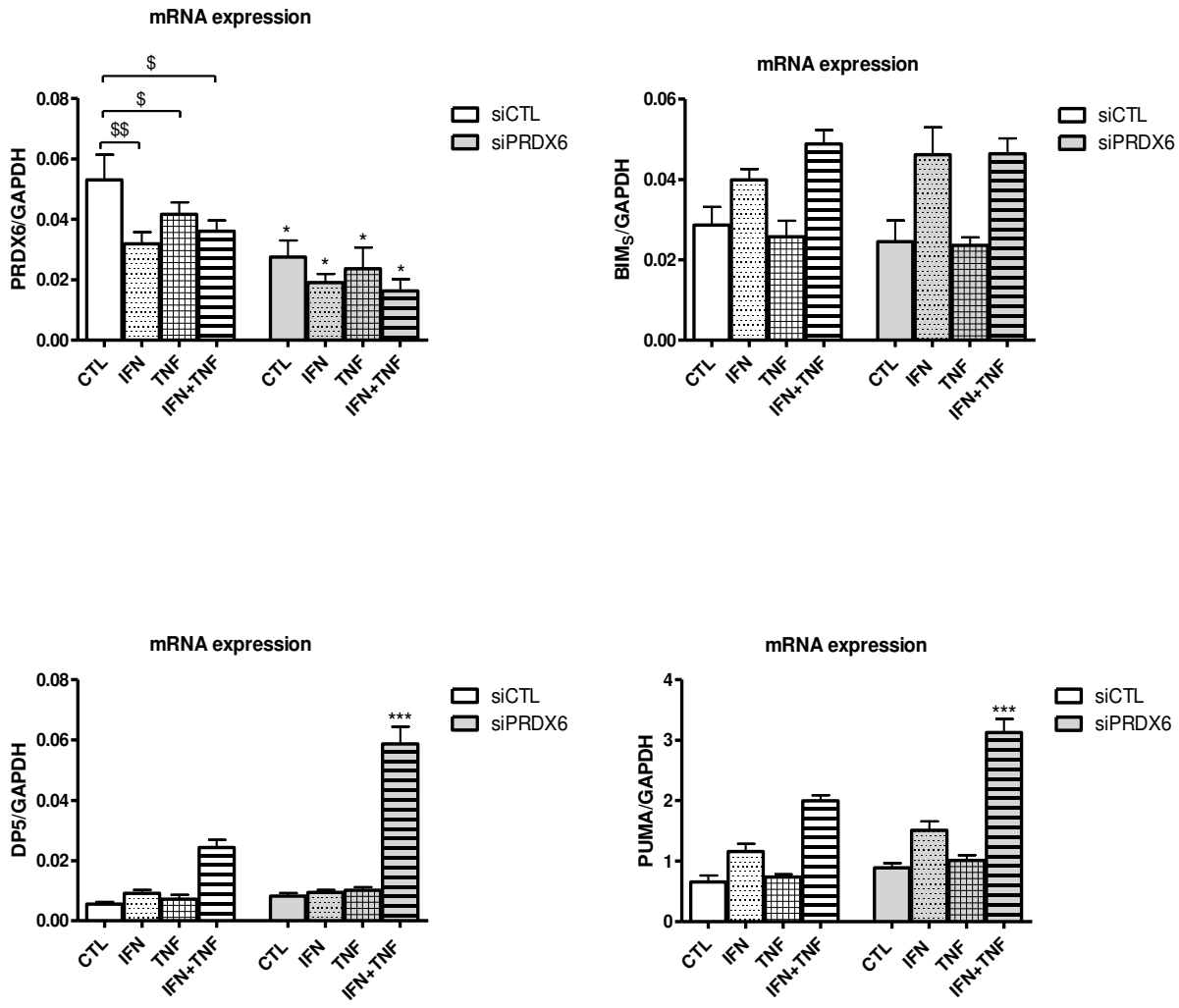
Os resultados mostrados abaixo se referem à modulação da PRDX6 pelas citocinas pró-inflamatórias, mas não foram utilizados no artigo apresentado anteriormente.

Figura 1



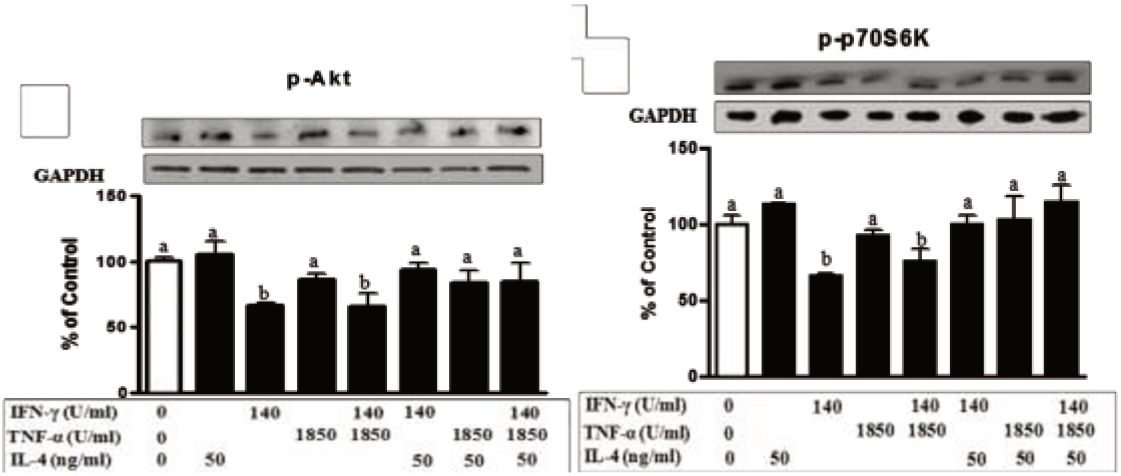
Efeito do KD da PRDX6 sobre a expressão proteica de Caspase-9 e -3 Clivada. Células RINm5F e INS1-E controle e KD para PRDX6 foram incubadas com 140U/ml de IFN-gamma e 1850 U/ml de TNF-alpha por 24 horas. Após esse periodo as células foram lisadas e utilizadas em análises de Western-blot. Os blots acima são representativos de 4 experimentos independentes, normalizados pelo controle interno GAPDH. Os resultados estão representados como média ± S.E.M. letras diferentes indicam diferença estatística. $P<0.05$. Two-way ANOVA seguido de Bonferroni.

Figura 2



Efeito do KD da PRDX6 sobre a expressão de mRNA de BIMs, DP5 e PUMA. Células INS1-E controle e KD para PRDX6 foram incubadas com 140U/ml de IFN-gamma e 1850 U/ml de TNF-alpha por 24 horas. Após esse periodo as células foram lisadas e utilizadas em análises de PCR em tempo real. Os resultados estão representados como média ± S.E.M, normalizados pelo controle interno GAPDH. n=4, $p<0.05$. Two-way ANOVA seguido de teste-t.

Figura 3



Expressão de p-Akt e p-70S6K após incubação das células RINm5F ao IFN-gamma, TNF-alpha e IL-4. Células RINm5F foram pré-incubadas com 50ng/ml de IL-4 por 24 horas e posteriormente incubadas com 140U/ml de IFN-gamma e 1850 U/ml de TNF-alpha por adicionais 24 horas. Após esse periodo as células foram lisadas e utilizadas em análises de Western-blot. Os blots acima são representativos de 4 experimentos independentes, normalizados pelo controle interno GAPDH. Os resultados estão representados como média ± S.E.M. letras diferentes indicam diferença estatística. $P<0.05$. Two-way ANOVA seguido de Bonferroni.