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"Possível papel do exercício físico como modulador da função de células beta pancreáticas: A importância da Interleucina-6 (IL-6) e da mobilização de L-arginina/glutamina"

TESE DE DOUTORADO

Orientador: Professor Dr. Paulo Ivo Homem de Bittencourt Júnior - UFRGS

Porto Alegre

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"Eu aprendi que pare se crescer como pessoa é preciso

me cercar de gente mais inteligente do que eu"

Willian Shakespeare

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SUMÁRIO

RESUMO

O presente trabalho será apresentado na forma das três publicações as quais deu origem. O primeiro trata-se de uma revisão que deu estrutura para a criação da hipótese e da justificativa de nosso trabalho com diabetes. Nesta revisão, criamos a hipótese de que a redução da disponibilidade de L-arginina para a célula beta, que é usada para produção de óxido nítrico para a secreção assistida de insulina, causa uma mudança no metabolismo da glutamina desviando-o da síntese de glutationa para a produção *de novo* de L-arginina, causando uma mudança redox que culmina na ativação do NF-κB exacerbando o processo inflamatório (insulite) e então morte celular. A partir desta hipótese testamos o papel do aumento da disponibilidade de L-arginina para uma célula clonal produtora de insulina (BRIN-BD11) sob influência de um coquetel sub-letal de citocinas pró-inflamatórias, mimetizando o ambiente real do inicio da insulite. Os resultados desta estratégia renderam observações interessantes: o aumento de L-arginina resultou em síntese de glutationa (GSH) e redução de dissulfeto de glutationa (GSSG), além de ter aumentado a expressão das heme-oxigenases (HO-1 e HO-3), conhecidas por suas ações anti-inflamatórias e anti-oxidantes, além de serem necessárias para a manutenção da função normal de células beta. Este tratamento ainda resultou em aumento no consumo de glicose e diminuição da produção de lactato, indicando que este aminoácido induziu o aumento das vias oxidativas e redução do metabolismo anaeróbio. A célula beta demonstrou apresentar uma significativa atividade basal da arginase e esta, juntamente com a NOS2, se mostrou induzível por citocinas pró-inflamatórias, fato importante para uma célula cuja função é dependente de óxido nítrico e apresenta baixas defesas antioxidantes, permitindo assim que a parte de L-arginina, por competição de substrato, seja desviada da síntese de óxido nítrico, evitando uma possível citotoxicidade. Estes resultados indicam que as células-beta necessitam de níveis basais de L-arginina para manter-se cosntitutivamente protegidas contra os efeitos das ações de citocinas pró-inflamatórias. No segundo trabalho investigamos o papel da IL-6, em concentrações similares as encontradas no exercício físico moderado (50μg/mL) na secreção de insulina e sinalização celular das mesmas células clonais (BRIN-BD11). De forma interessante, a IL-6 induziu o aumento na secreção de insulina tanto de forma crônica, quanto em condições basais e em situações de desafio metabólico (secreção estimulada pro glicose + alanina). Além disso o consumo de glicose foi aumentado em paralelo. Como mecanismos de ação para este aumento de sensibilidade induzido por IL-6, encontramos um nível aumentado de AMPK-p (forma ativa) juntamente com aumento de NOS2 e nitritos. Ambas as enzimas estão relacionadas com o controle metabólico, especialmente de ácidos graxos e, por esta razão sua ativação resultou em aumento de metabolismo e, consequentemente a secreção de insulina. A partir deste resultado, sugerimos que a IL-6 pode ser o ele de comunicação entre as células musculares e células beta pancreáticas durante e após o exercício, e que esta interação é importante para processos de adaptação e de diminuição da resistência a insulina.

1. INTRODUÇÃO

O diabetes tipo I resulta da destruição das células β secretoras de insulina das ilhotas de Langerhans do pâncreas por um processo mediado pelo sistema imunológico, causando hiperglicemia crônica e complicações como cegueira, insuficiência renal e neurodegeneração. Esta resposta imune adversa é induzida e promovida pela interação de fatores genéticos e ambientais, pertencendo a um grupo de doenças auto-imune que afeta cerca de 10% da população mundial de países desenvolvidos [1].

Acredita-se, que resposta auto-imune contra as células β das ilhotas, possa resultar de uma desordem da imunorregulação. De acordo com este conceito, as células T auxiliares (linfócitos T1, ou Th1), e suas citocinas produzidas (tipo 1) [Interleucina 2 (IL-2), Interferon Gama (IFN-γ), Fator de Necrose Tumoral Alfa (TNF-α)], sobrepujam as ações imunorregulatórias supressoras das células Th2 (outro subtipo de linfócitos T) e suas citocinas (tipo 2): IL-4, IL-10 e IL-6. Em teoria, isso permite que as citocinas do Th1 iniciem uma cascata de processos imune/inflamatórios nas ilhotas pancreáticas culminando na destruição das células β. O processo inflamatório decorrente é conhecido como insulite e é caracterizado pela infiltração de uma população mista de leucócitos nas ilhotas de Langerhans [1].

O controle da ativação e do equilíbrio entre a produção das citocinas anti e pró-inflamatórias pode ser modulado por diversos fatores como, por exemplo, o estresse psicológico. Recentemente nosso grupo sugeriu que a ativação do eixo simpatico-hormônio liberador das corticotrofinas (CRH) pelo estresse psicológico é capaz de induzir a superatividade das células Th1 [1]. Esta resposta pode determinar um grande aumento da utilização de glutamina e, consequentemente uma redução no fornecimento de L-arginina, aminoácido necessário para a produção fisiológica de óxido nítrico para a secreção normal de insulina. Uma vez que a disponibilidade de L-arginina é diminuída, o metabolismo da glutamina nas células beta, que antes era utilizado primariamente como substrato para o fornecimento de glutamato para a síntese de glutationa (GSH- principal antioxidante) é desviado para a síntese de novo de L-arginina, diminuindo a concentração de GSH e levando a célula a um desbalanço redox que culmina na ativação do fator nuclear κB, exacerbando o processo inflamatório e a citotoxicidade induzida pelo óxido nítrico [1]. Além disso, a disponibilidade de L-arginina no microambiente das células beta pode ter como causa, a

secreção de arginase pelos macrófagos infiltrados nas ilhotas de Langerhans (KRAUSE et al , 2009, Submetido).

Sendo o exercício físico moderado capaz de induzir mudanças no padrão de produção e liberação de citocinas em favor das anti-inflamatórias (tipo II), especialmente IL-6, é possível que a prática regular de exercícios exerça efeitos benéficos a células beta que podem protegê-la contra o início da resposta inflamatória que segue no diabetes tipo I. Além disso, o exercício é capaz de normalizar o suprimento de glutamina e, consequentemente de L-arginina para a circulação. Este efeito pode ser positivo no que concerne à restauração do fluxo de glutamina/glutamato para a síntese de GSH e de defesas antioxidantes [1].

Pelas razões expostas acima, o presente trabalho teve por objetivo demonstrar a papel da interleucina-6, assim como do aumento da disponibilidade de L-arginina, em células-beta produtoras de insulina.

1.1 OBJETIVOS

1.1.1 OBJETIVOS GERAIS

Determinar os efeitos de diferentes citocinas anti e pró-inflamatórias, assim como o efeito do aumento da disponibilidade de L-arginina, sobre o metabolismo e função de células beta produtoras de insulina (BRIN-BD11).

1.1.2 OBJETIVOS ESPECÍFICOS

1- Determinar o efeito da IL-6, em concentrações similares as encontradas no exercício físico aeróbio moderado, sobre a secreção de insulina, metabolismo, estado redox e sinalização celular de uma célula clonal produtora de insulina (BRIN-BD11).

2- Determinar o efeito do aumento da disponibilidade de L-arginina sobre a secreção de insulina, metabolismo, estado redox e sinalização celular das células BRIN-BD11.

1.2 FORMATO DA TESE

A presente tese de doutorado será apresentada com base nos artigos publicados e submetidos a periódicos internacionais reconhecidos, sendo:

1- Revisão de literatura e hipóteses deste trabalho: "Type 1 diabetes: can exercise impair the autoimmune event? The L-arginine/glutamine coupling hypothesis" (artigo publicado na revista *Cell Biochemistry and Function, 2008*);

2- Estudo 1: "The effect of IL-6 on insulin secretion, glucose consumption, nitric oxide release, signal transduction and redox status in a clonal pancreatic β-cell line: a possible cytokine-mediated cross-talking between skeletal muscle and pancreatic β-cells?" (artigo submetido para a revista *The Pflugers Archiv-European Journal of Physiology, 2009*);

3- Estudo 2: "Does the L-arginine availability determine the beta-cells fate during the insulitis?" (artigo submetido para a revista *Diabetes, 2009*).

2. REVISÃO DE LITERATURA E HIPÓTESES DESTE TRABALHO: Type 1 diabetes: can exercise impair the autoimmune event? The L-arginine/glutamine coupling hypothesis

REVIEW ARTICLE

Type 1 diabetes: can exercise impair the autoimmune event? The L-arginine/glutamine coupling hypothesis

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Prevention of type 1 diabetes mellitus (T1DM) requires early intervention in the autoimmune process directed against β-cells of the pancreatic islets of Langerhans, which is believed to result from a disorder of immunoregulation. According to this concept, a T-helper lymphocyte of type 1 (Th1) subset of T-lymphocytes and their cytokine products, the type 1 cytokines [*e.g.* interleukin 2 (IL-2), interferon gamma (IFN-γ) and tumour necrosis factor beta (TNF-β)] prevail over immunoregulatory (anti-inflammatory) Th2 subset and its cytokine products, *i.e.* type 2 cytokines (*e.g.* IL-4, IL-6 and IL-10). This allows type 1 cytokines to initiate a cascade of immune/inflammatory processes in the islet (insulitis), culminating in β-cell destruction. Activation of sympathetic-corticotropin-releasing hormone (CRH) axis by psychological stress induces specifically Th1 cell overactivity that determines enhanced glutamine utilization and consequent poor L-arginine supply for nitric oxide (NO)-assisted insulin secretion. This determines the shift of intraislet glutamate metabolism from the synthesis of glutathione (GSH) to that of L-arginine, leading to a redox imbalance that activates nuclear factor κB exacerbating inflammation and NO-mediated cytotoxicity. Physical exercise is capable of inducing changes in the pattern of cytokine production and release towards type 2 class and to normalize the glutamine supply to the circulation, which reduces the need for glutamate, whose metabolic fate may be restored in the direction of GSH synthesis and antioxidant defence. Also, the 70-kDa heat shock protein (hsp70), which is immunoregulatory, may modulate exercise-induced anti-inflammation. In this work, we envisage how exercise can intervene in the mechanisms involved in the autoimmune process against β-cells and how novel therapeutic approaches may be inferred from these observations. Copyright © 2008 John Wiley & Sons, Ltd.

KEY WORDS — diabetes; cytokines; arginine metabolism; glutamine metabolism; islet β-cells; exercise

ABBREVIATIONS — ALR/Lt and ALS/Lt, alloxan resistant (and sensitive) mouse strain; Akt/PKB, v-Akt/protein kinase B; AL, alloxan; AMPK, adenosine monophosphate-dependent protein kinase; CAT, catalase; COX-2, inducible cyclooxygenase-2; CP-PG, cyclopentenone prostaglandin; CRH, corticotropin-releasing hormone; GSH, glutathione, reduced form; GSPx, glutathione peroxidase; GSR, dglutathione disulphide reductase; HSF, heat shock transcription factor; hsp70, 70-kDa heat shock protein; IκB, inhibitor of NF-κB; IFN, interferon; IKK, IκB-directed kinase; IL, interleukin; IL-1ra, interleukin-1 receptor antagonist; iNOS, inducible form of nitric oxide (NO) synthase; JAK, Janus non-receptor tyrosine kinase; JNK, c-Jun N-terminal kinase; Keap1, Kelch-like ECH-associated protein 1; LPS, bacterial lipopolysaccharide; MAPK, mitogen-activated protein kinase; MHC, major histocompatibility gene complex; NF-κB, nuclear factor of κB family; NK, natural killer; NO, nitric oxide; NOD, non-obese diabetic; Nrf2, nuclear factor-erythroid 2 p45-related factor 2; NT, nitrotyrosine; O_2^-, superoxide anion; ONOO$^-$, peroxynitrite; phox (also known as NAD(P)H oxidase), phagocyte oxidase; ROS/RNS, reactive oxygen/nitrogen species; SOD, superoxide dismutase; STAT, signal transducer and activator of transcription; STZ, streptozotocin; T1DM, type 1 diabetes mellitus; Th1, 2 or 3, T-helper lymphocyte of type 1, 2 or 3; TNF, tumour necrosis factor.

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INTRODUCTION

Type 1 diabetes mellitus (T1DM) is a chronically progressive autoimmune disease that affects *ca.* 10% of the population in the developed world. This adverse immune response is induced and promoted by the interaction of genetic and environmental factors. Although T1DM may be much more prevalent in children and adolescents, the former nomenclature 'childhood' or 'juvenile' diabetes or 'insulin-dependent' diabetes mellitus (IDDM) has largely been abandoned, since not everyone with autoimmune diabetes is either a juvenile or necessarily exhibits an absolute insulin requirement.[1]

The main feature of T1DM autoimmune process is an inflammatory response targeted specifically at β-cells in the islets of Langerhans thus causing their mass reduction and dysfunction. An islet autoimmune response is mediated, basically, by the T-helper lymphocyte of type 1 (Th1) subset of T-lymphocytes and their cytokine products, *i.e.* the type 1 cytokines [*e.g.* interleukin-2 (IL-2), interferon-γ (IFN-γ) and tumour necrosis factor-β (TNF-β)], which prevail over the immunoregulatory Th2 subset and its cytokine products, the type 2 cytokines (mainly, IL-4, IL-6 and IL-10). This results in insulitis and β-cell destruction.[2]

Although insulin replacement may ameliorate the symptoms of the endocrine disease, it nevertheless does not affect the autoimmune process. Hence, preventing the progression of T1DM in its early stages requires an action over the autoimmune processes of the disease. Accordingly, initial trials for blocking the progression of early diabetes have used immunosuppressive agents, including prednisone,[3] azathioprine,[4] anti-thymocytic globulin[5] and cyclosporine A[6] in the hope that these agents would suppress the islet cell inflammatory process induced by pathogenic T-cells. However, these treatments have been shown to result in only a modest delay of diabetes in a few patients. No long-term effects have been observed and discontinuation of the drugs causes the autoimmune process to recur. In addition, these agents usually show significant toxicity.

On the other hand, recent studies from this laboratory have suggested that physical exercise may interfere with immune system function even at low intensity and duration.[7] Moreover, considering its powerful ability to modulate oxidative stress and protect against chronic inflammatory conditions, physical exercise has emerged as a possible effective therapy against the development of T1DM. In particular, exercise effects on the cytokine response that may result in anti-inflammation and β-cell protection are remarkable.[8] Therefore, in this article, we argue that mechanisms of cytokine response to exercise may oppose unfavourable immune responses during T1DM initiation and or progression. Exercise-elicited metabolic changes that drive glutathione (GSH)-based antioxidant protection in β-cells are also examined.

UNDERLYING MECHANISMS OF THE T1DM AUTOIMMUNE PROCESS

A large body of evidence reinforces the notion that, during the onset of T1DM, β-cells are destroyed by an autoimmune response directed against specific β-cell constituents called autoantigens.[9] Interestingly, T-cells specifically reactive to islet β-cell autoantigens do exist normally, but they are restrained by immunoregulatory mechanisms, collectively known as the 'self-tolerant state'.[10] It has been postulated that T1DM may develop when at least one of the immunoregulatory mechanisms fails, allowing auto-reactive T-cells directed against β-cells to become activated, thus leading to a cascade of immune/inflammatory processes in the islet that is termed 'insulitis'. This, eventually, leads to the loss of most β-cell mass after prolonged periods of the disease.[11] β-Cell death in the course of insulitis is probably caused by direct contact with activated macrophages and T-cells, and thus to exposure to soluble mediators secreted by these cells, including cytokines, oxygen free radicals and nitric oxide (NO), which is also a free radical ($\cdot$N=O).[12]

It is currently hypothesized that the 'pathogenic' immune response is mediated by a Th1 lymphocyte subset, whereas the 'protective' immune response is mediated by a Th2 subset.[13] Th1 and Th2 cells are characterized by distinct cytokine secretory products.[14] Accordingly, Th1 cells secrete IL-2, IFN-γ, IL-12 and TNF-β (collectively known as type 1 cytokines), whereas Th2 secrete IL-4, IL-6 and IL-10 (type 2 cytokines), amongst others. Also, different Th cell phenotypes, other than Th1 and Th2, have also been demonstrated, having distinct patterns of cytokine secretion (*e.g.* Th3 cells, which produce TGF-β). Th1 and Th2 cell subsets and their distinct cytokine products may lead to extremely different T-cell actions[14–16]: Th1 cells and their cytokines are the physiological mediators of cell-mediated immunity, while Th2 cytokines are much more effective stimulators of humoral immune responses such as immunoglobulin production. Th1-derived cytokines can activate vascular endothelial cells to recruit

circulating leukocytes into the site of antigen stress, whereas they activate macrophages to eliminate the antigen-bearing cell. Furthermore, Th1-derived IL-2 and IFN-γ activate cytotoxic T cells to destroy target cells expressing the appropriate major histocompatibility gene complex (MHC)-associated antigen and also activate natural killer (NK) cells to eliminate target cells in an MHC-independent manner, so that Th1 cytokines activate cellular immune responses. Interestingly, the responses of Th1 and Th2 cells are mutually inhibitory. Accordingly, Th1 cytokines (*e.g.* IFN-γ) are able to inhibit the production of Th2 cytokines (*e.g.* IL-4, IL-10); these mediators, in turn, inhibit Th1 cytokine production.[14–16] As depicted in

Figure 1, a Th function imbalance tending to an auto-reactive Th1 prevalence over Th2 cells may trigger inflammation-dependent cell death and insulitis.

A variety of mechanisms have been proposed to explain Th1-cytokine cytotoxic effects on islet β-cells. Most current evidence points to oxygen and nitrogen free radicals as mediators of cytokine-induced islet β-cell destruction.[17–19] The precise mechanisms of β-cell destruction in T1DM remain to be demonstrated, however.

The non-specific immunoinflammatory response involves β-cell destruction by T-cell and macrophage-derived molecules, pro-inflammatory cytokines (IL-1, TNF-α, TNF-β, IFN-γ), hydrogen peroxide (H_2O_2)
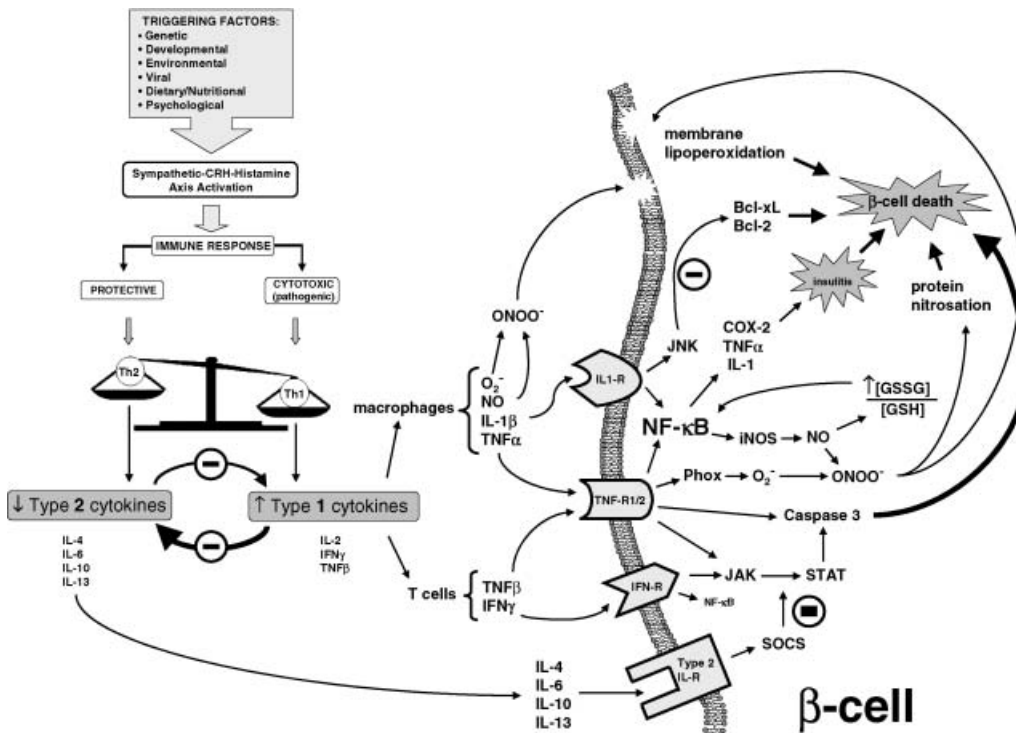

Figure 1. Triggering mechanisms involved in the autoimmune diabetes. Different stressful situations may lead to the activation of sympathetic-corticotropin-releasing hormone (CRH)–histamine axis that triggers a Th1-specific immunoinflammatory response. Auto-reactive Th1 cell subset and its cytokine products (type 1 cytokines) raised against islet β-cell antigen(s) mediate the activation of macrophages and Th lymphocytes. Although type 1 cytokines may differently activate β-cell signaling cascades, NF-κB-mediated responses occupy a critical position in favoring insulitis by dictating the expression of a number of inflammatory enzymes and mediators. iNOS-dependent overproduction of NO is central to this process by shifting the redox potential ([GSSG]/[GSH] ratio) towards an oxidative state (which feeds forward NF-κB) that may lead to lipoperoxidation and disruption of membrane integrity. The binding of TNF-α to its receptor (TNF-R1) or to TNF-β receptor (TNF-R2) determines NAD(P)H oxidase/phox activation and massive production of O_2^- which promptly reacts with NO to form the strong oxidant agent peroxynitrite ($ONOO^-$). Activated T-cell-derived TNF-β is also capable of cross-reacting with either TNF receptor leading to the same effect. Additionally, TNF-R1/2 and IFN-γ receptor (IFN-R) ultimately turn on the caspase-3 apoptotic pathway, whereas IL-1-dependent activation of the JNK cascade shuts off the Bcl-xL/Bcl-2 anti-apoptotic pathway culminating in β-cell death that determines T1DM. Conversely, Th2 subset and several Th2-dependent type 2 cytokines (*e.g.* IL-4, IL-6, IL-10 and IL-13) tend to be protective by blocking JAK/STAT-mediated apoptosis via the SOCS-dependent pathway that is activated by type 2 IL receptors. Despite the fact that Th1 and Th2 responses are mutually inhibitory, evidence suggests that factors predisposing to insulitis causes a Th cell imbalance favoring Th1 priming, which is a determinant for the onset of T1DM

and free radicals such as superoxide (O_2^-, called $O_2 \cdot^-$, hereafter) and NO. These inflammatory mediators are produced by T-cells (both $CD4^+$ and $CD8^+$) and T-cell cytokine-activated macrophages.[15] Interestingly, mRNA levels of inducible form of nitric oxide synthase (iNOS) directly correlate with IL-1α and IFN-γ mRNA in islets of pre-diabetic NOD (non-obese diabetic) mice, while iNOS protein is expressed in islet-infiltrating macrophages and in β-cells themselves.[20] Hence, IL-1 (from macrophages) and IFN-γ (from T-cells) induce an increase in iNOS protein expression and activity which is accompanied by NO production from macrophages and β-cells (Figure 1). Indeed, generation of NO within Langerhans islets is an important trigger of β-cell damage by activated macrophages[21]: macrophage-derived NO is destructive to adjacent β-cells, and β-cell-derived NO is self-destructive at high concentrations. Also, intra-islet release of IL-1 by the activation of passenger macrophages *in vitro* leads to iNOS expression within β-cells and consequent impaired insulin secretion.[22]

OXIDATIVE STRESS-MEDIATED β-CELL DESTRUCTION

Oxidative stress is believed to play an important role in the development of T1DM and its subsequent complications.[23] Current evidence points to oxygen and nitrogen free radicals as major mediators of cytokine-induced β-cell destruction.[17] As discussed below, β-cells are highly vulnerable to injury by free radicals, such as those produced by alloxan (AL)[24] and streptozotocin (STZ),[25] which has been attributed, at least in part, to the low activities of oxygen free radical scavenging enzymes in islet β-cells, especially mitochondrial manganese-type superoxide dismutase (Mn-SOD),[26] glutathione peroxidase (GSPx)[24,27] and glutathione disulphide (GSSG) reductase (GSRd).[27] Also, the expression of mRNA encoding for several antioxidant enzymes, such as Mn-SOD, cytoplasmic copper–zinc-type SOD (Cu/Zn-SOD), GSPx and catalase (CAT), has been reported to be lower in islets of Langerhans compared with other mouse tissues.[28] Additionally, the administration of antioxidants (nicotinamide, SOD, α-tocopherol, probucol and Lazaroid), as well as oxygen free radical scavengers, has been used *in vitro* to protect islets from the cytotoxic effects of some pro-inflammatory cytokines (IL-1, TNF-α and INF-γ), concurrently providing *in vivo* protection against the development of the autoimmune diabetes process.[29]

Conversely, studies on MnSOD and CAT transgenics have shown that protection of islets from oxidative stress does not alter cytokine toxicity,[30] which indicates that although related to each other, oxidative stress and cytokine-induced islet toxicity may use specific and diverse pathways to induce β-cell death downstream.

Incubation of rat[31] and human[32] islet cells with a cytotoxic combination of cytokines (IL-1, TNF-α and IFN-γ) has been reported as an inducing factor for lipid peroxidation (also known as lipoperoxidation). When individually administered, however, the same cytokines have been shown to inhibit insulin release without any increase in lipid peroxidation or cytodestructive effects in rat islets.[33] Taken together, these findings suggest that cytokine-induced inhibition of insulin release may not be oxygen free radical-mediated, whereas the cytodestructive effects of cytokines on β-cells do appear to involve free radical-mediated events that induce the formation of toxic aldehydes within the islets of Langerhans.[34] This strongly suggests that type 1 cytokines interfere in β-cell metabolism at some point that is intimately related to insulin secretion.

An additional complication to this scenario is the fact that β-cells express mitochondrial uncoupling protein 2 (UCP2) which dissipates the coupling between electron transport from ATP formation favouring O_2^- generation. Since O_2^- anion is a powerful activator of UCP2, a positive feedback mechanism exists in that O_2^- generation enhances its own formation. This is particularly critical under prolonged hyperglycaemia, where UCP2 activity may be extremely high thus worsening insulin secretion by β-cells[35] and may partially explain the exhaustion of β-cell function after long-term T2DM that, if untreated, evolves to T1DM. Furthermore, the high-glucose, high fatty-acid environment created by either insulin-deficiency or insulin-resistance favours the expression of NAD(P)H oxidase with consequently enhanced ROS production and β-cell death.[36]

The physiological nitrogen free radical NO is an extremely reactive gas that has been demonstrated to be a potent and pleiotropic mediator that fulfils a variety of different functions in a number of physiological and pathological processes in the human body. The production of NO by endothelial cells is necessary for the normal regulation of vascular tone and function. Its overproduction, on the other hand, plays a crucial role in the development of catecholamine-resistant hypotension during septic shock. Conversely, endothelial dysfunction in patients with atherosclerosis, essential hypertension and

diabetes mellitus is accompanied by a decreased basal endothelial NO production.[37] For instance, in a recent study, we have assigned a significant negative correlation between endothelium-derived NO and insulin resistance in polycystic ovary syndrome (PCOS) patients.[38]

The complex host response of immunocompetent cells involves local overproduction of NO that leads to the destruction of bacterial or viral agents.[39] A similar mechanism, however, may be responsible for the damage of some cells and tissues in a series of autoimmune diseases including T1DM.[18] Indeed, NO has been identified as an important mediator of β-cell damage induced by activated macrophages[21] and is considered a death effector in pancreatic β-cell death.[18] NO is physiologically produced in β-cells from L-arginine in a reaction catalysed by the iNOS (EC 1.14.13.39, encoded by *NOS-2* gene).[40] Additionally, iNOS-dependent NO production has been demonstrated to be a causal component of insulin resistance via the *S*-nitrosation of cysteine-containing proteins that are critical for insulin receptor signalling, including IRS-1 and Akt/protein kinase B (Akt/PKB).[41] Therefore, the possibility cannot be dismissed that immune system-dependent overaccumulation of NO may be destructive to β-cells also by the *S*-nitrosation of critical thiol groups located in signalling proteins and transcription factors that eventually promote β-cell death. However, studies on the effects of iNOS inhibitors in the rat insulinoma cell line RINm5F have suggested that NO production is not sufficient for cytokine-induced destruction of β-cells.[2] In fact, NO-independent free radical-mediated cytotoxic mechanisms seem to be involved as well.[42] Similarly, IL-1β, TNF-α and IFN-γ have been shown to suppress glucose-stimulated insulin release and mediate destruction of human islet cells irrespective of their effects on NO generation.[43]

Peroxynitrite (ONOO⁻), which is in equilibrium with its conjugate peroxynitrous acid (ONOOH, $pK_a \approx 6.8$),[44] is a highly reactive oxidant species produced by the combination of the oxygen free radical O_2^- and NO[45] and has been demonstrated to be a more potent oxidant and cytotoxic mediator than NO or O_2^- individually, in a variety of inflammatory conditions.[46] ONOO⁻ is extremely cytotoxic to rat and human islet cells *in vitro*[47] and its *in vivo* formation has recently been reported in pancreatic islets where it has been associated with β-cell destruction and development of T1DM in NOD mice.[2] Detection of nitrotyrosine (NT), a marker of *in vivo* ONOO⁻ formation, in islet-infiltrating macrophages, as well as in β-cells of pre-diabetic

NOD mice, has also been provided by immuno-histochemical studies, which suggests that both oxygen and nitrogen free radicals could mediate β-cell destruction in autoimmune diabetes.

A potential target for reactive oxygen/nitrogen species (ROS/RNS) is the nuclear transcription factor of κB family (NF-κB). It is noteworthy that NF-κB was the first redox-sensitive eukaryotic transcription factor shown to respond directly to oxidative stress in many types of cells[48] while its activation leads to the expression of at least a hundred of inducible proteins directly involved in inflammation, such as inducible cyclooxygenase-2 (COX-2), iNOS, TNF-α and IL-1.[49] Therefore, NF-κB is, at the same time, both a target and an inducer of inflammation and inflammation-induced oxidative stress. In resting (unstimulated) cells, NF-κB dimeric complexes are predominantly found in the cytosol where they are associated with members of the inhibitory IκB (inhibitor of NF-κB) family,[49] so that NF-κB gene products are entirely inducible proteins whose activation is dictated by specific stimuli that activate IκB-directed kinase (IKK) complexes. These stimuli include high intra-cellular GSSG levels and oxidative stress *per se*.[48] IKKs, in turn, phosphorylate IκB proteins directing them to proteasome-mediated degradation, which sets NF-κB dimers free to bind to DNA in the nucleus. NF-κB activation is responsible for both initiation and amplification of immune and inflammatory responses in all cells. Actually, NF-κB activation is *sine qua non* for the control of immune and inflammatory responses,[50–52] and since inflammatory factors, such as pro-inflammatory cytokines, chemokines, adhesion molecules, colony-stimulating factors and inflammatory enzymes, are NF-κB-dependent gene products, dysregulation or aberrant activation of NF-κB could initiate inappropriate autoimmune and inflammatory responses. Conversely, inhibition of NF-κB activation has been argued as a potential therapeutic approach in several immune and inflammatory-related diseases.[53] This is why cyclopentenone prostaglandins (CP-PGs), which are powerful inhibitors of NF-κB activation,[54] are now considered to be the physiological mediators of the 'resolution of inflammation',[55] whereas CP-PG-based pharmacological approaches (*e.g.* Lipo-Cardium technology) have proved to be strong anti-atherosclerotic strategies.[55–57]

NF-κB is a redox-sensitive transcription factor and different agents known to induce oxidative stress, such as phorbol esters, inflammatory cytokines, UV light, X- and γ-rays, viral and bacterial proteins and lipopolysaccharide (LPS), trigger NF-κB activation.[58,59] Contrarily, NF-κB activation can be

inhibited by addition of various antioxidants including lipoic acid,[60] N-acetylcysteine,[61] vitamin E derivatives,[62] pyrrolidine dithiocarbamate,[63] selenoproteins,[64] and vitamin C.[65] Dietary antioxidant supplementation to animals has also been shown to inhibit NF-κB activation. ROS directly signalize the release and degradation of IκBα, which results in a rapid translocation of the active NF-κB towards the nucleus.[50,66,67] Furthermore, antioxidants reduce NF-κB activation by ROS, but cannot alter NF-κB activation by cytokines, as measured by IκB phosphorylation, NF-κB translocation, iNOS and NO production.[68] Therefore, ROS-mediated activation of NF-κB is a key event in the destruction of β-cells and, consequently, to the development of T1DM.[69] Moreover and remarkably, however, β-cell specific inhibition of NF-κB activation completely protects mice against STZ-induced diabetes leading to reduced apoptosis and NO production *in vitro* and diminished intraislet lymphocytic infiltration *in vivo*.[70] As a corollary, an imbalance between ROS production and antioxidant defences may induce NF-κB activation and inflammation, which, in turn, amplifies ROS/RNS production leading to β-cell destruction, hyperglycaemia and the development of T1DM.

In many tissues, ROS and RNS are generated as a result of the infiltration of immune cells that produce these mediators as an intrinsic defensive mechanism used to damage and kill pathogenic organisms.[71] On the other hand, inflammatory cytokines activate NF-κB, amplifying ROS production, thus creating a vicious cycle. For instance, the binding of TNFα to its membrane receptors (TNF-R1 and TNF-R2) in target cells turns on NAD(P)H oxidase/phox (for phagocyte oxidase, which produces O_2^- from the molecular O_2) while concomitantly activating NF-κB-dependent iNOS expression. This leads to a massive production of NO,[72] which, in turn may promptly react with O_2^- to produce the extremely toxic oxidant $ONOO^-$. In fact, additionally to the well-accepted role of mitochondrial electron transport chain-derived ROS, recent evidence indicates that ROS generated by NAD(P)H oxidase inside β-cells could contribute to the inhibition of insulin secretion, impaired signal transduction and cell death.[35] It is noteworthy that β-cells express at least three isoforms of phagocyte-like NAD(P)H oxidases.[73] Conversely, oxidative stress induced in inflamed tissues leads to the modification of critical cysteines present in Keap1 (Kelch-like ECH-associated protein 1), which activates Nrf2 (nuclear factor-erythroid 2 p45-related factor 2) and the eventual transcription of NAD(P)H

oxidase/phox, for phagocyte oxidase).[74,75] The cytosolic regulatory protein Keap1 is an inhibitory protein that, under basal conditions binds tightly to Nrf2, retaining it in the cytoplasm, so that Keap1 much resembles IκB family components that keep NF-κB dimers out of the nucleus.[75,76] Hence, in the development of T1DM, ROS/RNS may regulate both NF-κB and Nrf2 activation in the pancreas. The balance between these two pathways is therefore crucial for the initiation and propagation of the inflammatory and autoimmune processes responsible for β-cell death.[77,78]

Inflammatory cytokines, such as IL-1β, activate NF-κB in rodent and human islet cells,[12] while prevention of NF-κB activation protects pancreatic β-cells against cytokine-induced apoptosis.[79,80] A total of 66 cytokine-responsive NF-κB-dependent genes have been currently identified in β-cells, including genes encoding for cytokines and Inos.[81] Remarkably, the expression of *ca.* 50% of the β-cell genes that may be modified after cytokine exposure is secondary to iNOS-mediated NO formation.[82] It is of note that treatment of human, as well as rodent, β-cells with purified IL-1β alone is not sufficient to induce apoptosis, but if IL-1β is combined with IFN-γ, β-cells undergo apoptosis after few days in culture.[12] This suggests that an intracellular IFN-γ signal must synergize with IL-1β signalling pathways in order to trigger β-cell apoptosis (Figure 1). IFN-γ binds to cell surface receptors and activates the Janus tyrosine kinases JAK1 and JAK2. These kinases phosphorylate and activate their downstream transcription factor STAT-1 (for signal transducers and activators of transcription), which dimerizes and translocates to the nucleus where it binds to γ-activated sites on target genes.[12] STAT-1 mediates the potentiating effect of IFN-γ on IL-1β-induced iNOS expression.[81] Because excessive activation of JAK/STAT signalling may lead to cell death, STAT transcriptional activity is regulated by multiple negative feedback mechanisms. These include dephosphorylation of JAK and cytokine receptors by cytoplasmic protein-tyrosine phosphatases SHPs (for Src homology 2 domain phosphatases), and inhibition of JAK enzymic activities by the suppressors of cytokine signalling (SOCS) family. Upregulation of either SOCS-1 or SOCS-3 protects β-cells against cytokine-induced cell death *in vitro* and *in vivo*.[83,84] SOCS-3 also protects insulin-producing cells against IL-1β-mediated apoptosis via NF-κB inhibition.[85] Evidence indicates that the fate of β-cells, after cytokine exposure, depends on the duration and severity of perturbation of key β-cell gene networks.

In addition to immunological factors, accidental chemical induction of diabetes, particularly via N-nitrous compounds, is well documented in humans.[86] It has been demonstrated that poor nutritional status as well as high-nitrite and/or high-nitrate diets are closely correlated with an increased incidence of T1DM.[87–89] Poor nutritional status produces an environment in which antioxidant defences are often low thus resulting in a higher susceptibility to oxidative damage. Taken as a whole, the above findings suggest that cytokine-elicited ROS/RNS production within β-cells may take part in an intricate cascade of activation of different gene products that culminate in β-cell destruction and onset of T1DM.?

β-CELLS: WHY SO MUCH SUSCEPTIBILITY TO OXIDATIVE STRESS?

Patients with T1DM exhibit major defects in antioxidant protection compared with healthy, non-diabetic controls. A significant reduction in total antioxidant status in both plasma and serum samples from T1DM patients is typically observed.[90] Diabetic children show significant reduction in GSH and GSPx in erythrocytes, as well as in plasma α-tacopherol and β-carotene levels.[91] In addition and notably, β-cells present a very low level of expression of antioxidant enzymes such as CAT and GSPx compared with other tissues, so that β-cells are prone to oxidative stress, particularly because this cell type shows a very accelerated mitochondrial flux of electrons (and consequently, elevated tendency towards ROS production) during glucose-stimulated insulin release.[35] Such a reduction in antioxidant activity is associated with significant increases in lipid hydroperoxides, conjugated dienes and protein carbonyls, which are markers for oxidative stress.[92] Moreover, a growing body of evidence indicates that, in the pre-diabetic condition, the antioxidant status appears to be impaired.[93] Hence, the low antioxidant defence in certain individuals (even if transiently) may predispose to an enhanced oxidative stress and the eventual β-cell death that categorize the onset of T1DM.

In vivo studies have also been performed in animal models that confirm the previous suspicions. AL and STZ are pancreatic β-cell selective toxins that have been extensively used to probe the mechanisms underlying oxygen-mediated damage to rodent β-cells. Both drugs reduce the level of NADH in pancreatic islets and inhibit proinsulin synthesis.[94,95] AL is also a hydrophilic unstable compound that is spontaneously and enzymically reduced by GSH, via a thiol transferase, or by NADPH, through cytochrome P450 reductase, within β-cells.[96,97] These redox reactions generate, in the presence of an iron catalyst, O_2^-, H_2O_2 and hydroxyl free radical (˙OH), which, ultimately may be a mediator of β-cell death and T1DM.[97,98] Similarly to that observed in humans (above), β-cell death induced by either AL or STZ in rodents can be limited or inhibited by the use of antioxidants (e.g. vitamin E) or pro-antioxidants (e.g. selenium, zinc), whereas dietary depletion of such antioxidants enhances the development of diabetes.[99] Also, pre-treatment or concomitant intravenous administration of vitamin A, GSH or the antioxidant enzyme SOD has been shown to confer protection against both AL and STZ, whereas nicotinamide seems to be protective only if administered before either of the diabetogenic drugs.[100–104]

Interestingly, not all AL-treated mice necessarily develop β-cell toxicity and T1DM. In fact, differential susceptibility among various inbred mouse strains to AL- or STZ-induced β-cell toxicity has been recognized for over 40 years.[105] AL-resistant (ALR/Lt) and AL-sensitive (ALS/Lt) mouse strains have been specifically selected for their resistance or susceptibility to T1DM induced by a single dose of the drug.[106] Many studies have shown that treatment of mice or rats with either AL or STZ increases antioxidant enzyme expression and activities in response to free radical stress.[107,108] Genetic differences in the constitutive ability to dissipate ROS may underlie the differential susceptibility to AL or STZ, distinguishing the ALR/Lt from the ALS/Lt inbred strains. Although CAT overexpression alone may be ineffective in protecting β-cells, combined overexpression of CAT and SOD1 (encoding Cu/Zn-SOD) has been shown to nullify AL treatment.[109] It is of note that the spleen and brain of ALR/Lt, rather than the pancreas, exhibit higher CAT levels compared with the ALS/Lt strain, while ALR/Lt animals present higher systemic and pancreatic GSH contents.[24,96,110] While the constitutively high levels of GSH in ALR/Lt may explain a seemingly natural antioxidant-based defence against AL challenge,[27] the apparently surprising high CAT expression in the spleen of resistant mice may provide a clue to the immune differences between sensitive and resistant mouse breeds that could answer the susceptibility to autoimmune diabetes.

Paralleling the in vivo observations in this area, many in vitro studies have been performed by using animal (rat, mouse and hamster)-derived insulinoma β-cells that, as yet, have not precisely identified a lack of adequate antioxidant defence as the origin of β-cell

destruction.[111] Interestingly, among the various cell types present in the Langerhans pancreatic islets, β-cells are particularly susceptible to free radical-mediated damage. As already mentioned, some cytokines, such as IL-1β, TNF-α and IFN-γ, are capable of inducing free radical formation and lead to β-cell death. Incubation of mouse pancreatic islets *in vitro* with a combination of these cytokines leads to the formation of toxic nitrogen and oxygen radicals, most of which are generated within the β-cells, not exogenously.[112] On the other hand, islet viability after cytokine exposure can be significantly increased by scavenging oxygen radicals[31,32] or by inhibiting the generation of nitrogen-based radicals.[113] However, Lakey *et al.*[114] have also found that human islets, concomitantly treated *in vitro* with cytokines and the non-specific iNOS inhibitor L-N^G-monomethylarginine (L-NMMA), have shown decreased islet viability and insulin content, which has been associated with high levels of hydrogen peroxide, so that NO-independent oxidative stress may also contribute to β-cell damage. This may also suggest an NO participation in the process of insulin release.

Mouse β-cells have been assumed to be 'passive bystanders' of their own destruction in the presence of an activated immune system. The ALR/Lt mouse strain has provided an unusual, but revealing, exception. As stated above, ALR/Lt mice have a constitutive ability to dissipate ROS/RNS and this was shown to confer protection against either AL or STZ. In a comparative study with NOD, C3H and ALR/Lt mice, Mathews *et al.*[115] have found that combined cytokine treatment (IL-1β, TNFα and IFNγ) mediates loss of viability in both NOD- and C3H-, but not in ALR/Lt mouse-derived islets, due to the generation of O_2^-, nitrogen free radicals and $ONOO^-$, as evidenced by NT generation and nitrotyrosinylation of islet cells. This unique AL resistance is unpredictable, as previous reports have demonstrated that islets exhibit a reduced expression and low activity of many antioxidant enzymes.[28,116] Also, cytokine-treated NOD islets, but not ALR/Lt ones, have shown upregulation of NF-κB within 1 h of exposure, which is accompanied by altered kinetics of IκB degradation. Interestingly, unlike NOD islets, ALR/Lt islets do not express IKKβ mRNA or protein, although ALR/Lt-derived livers may present normal IKKβ mRNA and protein expression. This finding resembles those of Eldor *et al.*, using a transgenic mouse strain that expresses a degradation-resistant IκB specifically in β-cells and where a nearly complete protection against STZ-diabetes is observed.[70] Therefore, it is possible that ALR/Lt islets, as well as β-cells from resistant

human subjects, are unique in maintaining an intracellular redox environment that prevents normal IKKβ gene expression in the basal state.[28,116] Indeed, it has been well documented that both low and high-oxidative milieu impair NF-κB activation and DNA binding activity.[48] In contrast, NOD dendritic cells[117] and macrophages[118] express very high constitutive NF-κB activity, apparently due to an inability to downregulate IKKβ,[119] this being likely the case for NOD islets.

Because cytokine treatment affects intracellular levels of both GSH and GSSG, even in ALR/Lt islets, it is purported that ROS/RNS are produced in ALR/Lt islets in response to combined cytokine treatments that are, nevertheless, rapidly detoxified by the antioxidant enzymes GSRd and GSPx, which are highly expressed in ALR/Lt islets. This rapid dissipation of endogenous oxidant species, coupled with a suppressed degradation of IκB, would account for the blunted activation of NF-κB, thus leading to the inhibition of iNOS induction and NO production in ALR/Lt islets.[120,121]

Analysis of ALR/Lt heart mitochondria following either *in vivo* AL administration or after *in vitro* $ONOO^-$ exposure has shown that ALR/Lt mitochondrial proteins are protected from NT formation.[120] Similarly, the absence of cytokine-induced NT formation in ALR/Lt islets is associated with insufficient NO formation, failure in generating O_2^- and H_2O_2 in addition to increased GSRd and GSPx activities which may rapidly dissipate any ROS/RNS accumulation.[122,123] Actually, it is assumed that elevated basal GSH levels inside ALR/Lt islets may increase the susceptibility for the subsequent modification by GSPx thus allowing for the elimination of NT from proteins in these cells. Removal of NT residues is important not only to maintain protein functions[120,124] but also because it prevents neoantigen generation and consequent activation of non-tolerant T-cells.[125] In conclusion, ALR/Lt genome confers a systemic resistance to free radical stress that is manifested at the pancreatic islet level by a failure of pro-inflammatory cytokines to activate NF-κB and induce iNOS. The consequent concentrations of NO produced are associated with enhanced ease of detoxification against the limited ROS/RNS that are produced, which prevents nitrosylation of cellular proteins and β-cell death.[115]

Altogether, the above results clearly indicate that the extent to which an individual responds to inflammatory cytokines producing NO, as well as the ability to scavenge and detoxify endogenous free radicals and ROS/RNS as they are formed in

pancreatic β-cells, may be an important component of genetically inherited susceptibility or resistance to T1DM. Nonetheless, at the same time, if NF-κB-dependent iNOS-mediated NO production is so harmful to β-cells, a question is thus promptly raised as to why β-cells would have such a sophisticated self killing machinery? The following observations provide clues for the answer to this query.

It has long been known that L-arginine, the immediate precursor of NO, is one of the most potent secretagogues of insulin,[126] while L-arginine deficiency is associated with insulinopenia and failure to secrete insulin in response to glucose.[127] In addition to this, NO has been incontestably shown to be a physiological regulator of insulin secretion in β-cells, in an elegant experimental protocol designed by the Prof. Anne Marie Salapatek's group in Canada, in a seminal paper[40] that deserves deep and meticulous reading. They have also reported that endogenous NO production can be stimulated by glucose, and that this stimulation can be blocked by NOS inhibition, whereas scavenging of NO specifically blocks insulin release stimulated by physiological intracellular concentrations of NO-donors (2 mM), but has no effect on the release stimulated by elevated K^+. It has also been found that NO donation does not elicit a β-cell intracellular Ca^{2+} ($[Ca^{2+}]_i$) response by itself, but was able to potentiate a glucose-induced $[Ca^{2+}]_i$ response. Since NO is a strong haeme-reactant, it partially inhibits the mitochondrial respiratory chain by binding to cytochrome c and/or cytochrome oxidase. As a consequence, the mitochondrial membrane potential decreases and Ca^{2+} leaves the mitochondria. This is followed by restoration of the mitochondrial membrane potential and Ca^{2+} reuptake by mitochondria.[127] Therefore, overproduction of NO under massive inflammatory stimuli does merit worry, not NO production itself. As previously argued,[40] the precise level of NO is crucial in determining its resultant effect, with low levels being involved in physiological signalling and higher levels becoming cytotoxic.[128,129] Hence, the supraphysiological elevation of NOS substrate, or the application of exogenous NO donors under these conditions of already elevated NO, may result in excessive NO production, yielding cytotoxic effects.[40] This appears to be the case of immunologically stimulated iNOS-catalysed NO production.

From the above propositions, it seems clear that the development of T1DM is not simply a question of cytokine imbalance culminating in a redox disruption and consequent oxidative stress that kills β-cells. This, in fact, raises another doubt: would it be solely a question of unresolved antioxidant defence that somehow could not be solved during β-cell evolution? If this were be the case, it would appear preposterous that such a sophisticated cellular machinery remains so prone to endogenously-generated NO-mediated self-destruction. The intricate metabolism of L-arginine in β-cells may unravel some important points in this regard.

L-ARGININE METABOLISM AND ITS ROLE IN T1DM

In β-cells, pro-inflammatory cytokines induce the production of NO, synthesized from L-arginine, via a reaction catalysed by iNOS, whose functionality depends on NF-κB-driven gene transcription and *de novo* enzyme synthesis. iNOS also utilizes NADPH and O_2 as co-substrates, in a shunt from the urea cycle (Figure 2) and, physiologically, L-arginine is the limiting substrate for NO production. In addition to this, however, pancreatic β-cells express another L-arginine-metabolizing enzyme, *i.e.* L-arginase (L-arginine amidinohydrolase, EC 3.5.3.1), which allows for the completion of the urea cycle through the formation of L-ornithine and urea from L-arginine.[130] Actually, physiological levels of L-arginase gene expression and activity have been measured in rat β-cells and the insulin-secreting cell line RINm5F.[130–133] β-Cells express both the cytosolic (L-arginase I) and the mitochondrial (L-arginase II) isoforms of the enzyme. Therefore, under certain circumstances, a true competition may occur in that the activity of iNOS relative to L-arginase dictates either NO or urea production in the pancreas (Figure 2). Consequently, L-arginase may impair NO production by limiting the availability of L-arginine for iNOS catalysis.[134–136] This notion is supported by the finding that inhibition of L-arginase results in enhanced NO synthesis in cytokine-activated cells.[137,138]

It has been demonstrated that cytokine-elicited co-induction of both NO (iNOS) and urea (argininosuccinate synthetase and argininosuccinate lyase) metabolic pathways occurs in many cell types,[139–141] including β-cells,[142] *in vitro* as well as *in vivo*. L-arginase activity may be increased in peritoneal macrophages after exposure to LPS,[143] while wound and peritoneal macrophages convert L-arginine to L-citrulline and L-ornithine at comparable rates, indicating that both iNOS and L-arginase pathways are functioning.[144,145] In clonal β-cells, IL-1β increases L-arginase activity with concomitant increase in NO production,[130] which suggests a kind
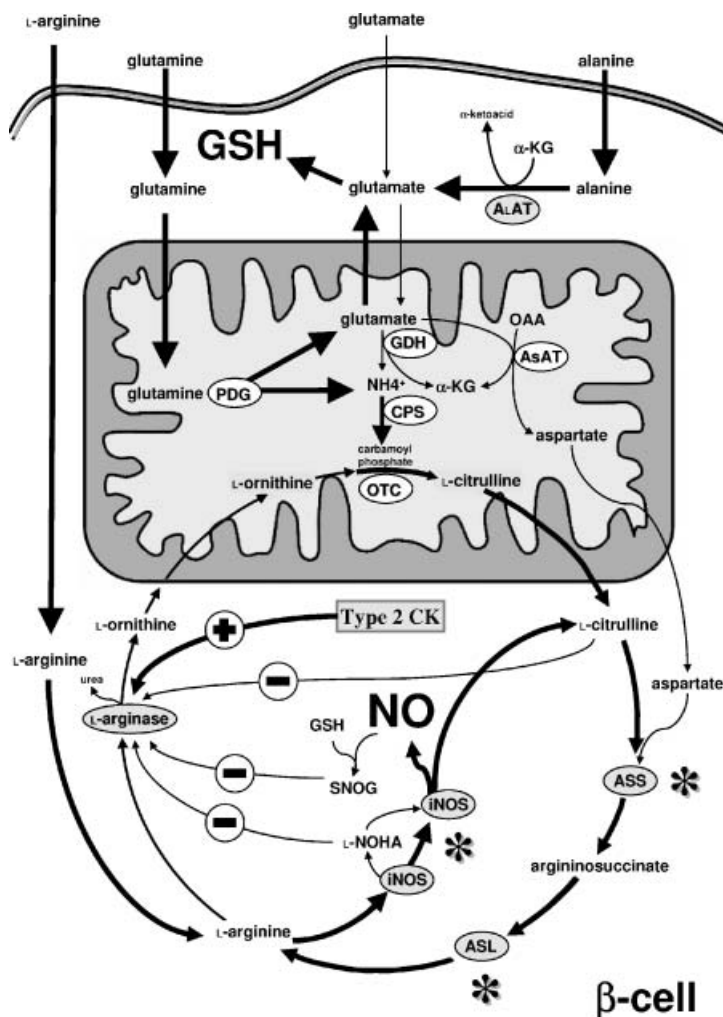
Figure 2.   Arginine–glutamine coupling and its role in T1DM. Under physiological secretagogue-mediated insulin release, both NO and GSH are obligatory intermediates. Accordingly, β-cells have an intricate iNOS-centred machinery to produce NO, which potentiates insulin secretion physiologically. At the same time, insulin-secreting pancreatic cells utilize glutamate-derived GSH in order to maintain redox status needed to allow hormonal secretion and to avoid a possible NO-mediated cytotoxicity. L-arginine derived from the kidney is the physiological substrate for the NF-κB-dependent iNOS-catalysed NO production in β-cells. Under insufficient L-arginine supply, however, the high throughput of NO for β-cells may be attained by the concerted action of phosphate-dependent glutaminase (GDP), glutamate dehydrogenase (GDH), aspartate aminotransferase (AsAT), carbamoylphosphate synthetase (CPS), ornithine transcarbamoylase (OTC), argininosuccinate synthetase (ASS) and argininosuccinate lyase (ASL), which, dramatically enhances the flux of glutamate towards NO production. Multiple negative feedback systems act in β-cells in order to warrant L-arginine entry in iNOS metabolic pathway. This is achieved mainly due to the inhibition of L-arginase activity by L-citrulline, N^G-hydroxy-L-arginine (L-NOHA, an intermediate in NO synthesis) and S-nitrosoglutathione (SNOG), which is formed during NO biosynthesis. On the other hand, β-cells have to synthesize GSH from glutamate, cysteine and glycine, because regeneration of GSH from glutathione disulphide (GSSG) via NADPH-dependent GSSG reductase is relatively low in β-cells because of the high flux of glucose towards ATP production that empty pentose-phosphate shunt, the major NADPH-producing system. In turn, *de novo* GSH synthesis is mainly dependent on liver-emanated supply of glutamate, which is not enough to allow for the enormous flux towards γ-glutamylcysteine synthetase and GSH synthetase in the GSH biosynthetic pathway. Therefore, muscle-derived alanine and glutamine constitute the principal sources of glutamate for GSH synthesis. Because of this, any reduction in L-arginine supply to β-cells accounts for a rapid shift in glutamate metabolism from GSH synthesis towards NO production. For instance, during Th1-elicited immune responses, the concerted enhancement of NF-κB-mediated ([*]) expression of ASS, ASL and iNOS dramatically boosts NO production from glutamate. If this rise in NO production is not accompanied by an enhanced L-arginine supply to β-cells, NO becomes very cytotoxic. Type 2 cytokines (CK) may alleviate NO toxicity by enhancing L-arginase expression that deviates L-arginine to the formation of L-ornithine and urea

of coordinated regulation of L-arginase and iNOS in these cells.

There is also evidence for a reciprocal regulation of NOS and L-arginase during immune responses via the antagonistic effects of cytokines released from Th1 and Th2 cells. While L-arginase activity may be induced by the 'anti-inflammatory' Th2 cytokines IL-4, IL-10 and IL-13,[146–149] the Th1-derived 'pro-inflammatory' cytokine IFN-γ increases iNOS expression and activity, both alone and in synergy with other pro-inflammatory cytokines, such as IL-1β and TNF-α.[150] Reciprocal effects of Th1- and Th2-derived cytokines on L-arginase and iNOS activities have also been shown by the treatment of murine macrophages with cytokines,[146,151] and by co-culturing murine macrophages with Th1 and Th2 T-cell clones.[152] In mouse bone marrow-derived macrophages, iNOS and L-arginase activities are regulated reciprocally by Th1 and Th2 cytokines, a strategy that guarantees a precise and efficient production of NO.[146]

Because of the above statements, a Th1/Th2 dichotomy has been proposed to play a central role in the pathogenesis of T1DM,[10] whereas evidence suggests that the progression of the disease correlates with a Th1-type immune response.[143–145,153] Increased generation of NO following cytokine-elicited iNOS induction during insulitis may contribute to β-cell destruction.[146,154] Therefore, competition between L-arginase and iNOS may be particularly important in protecting β-cells against the establishment of T1DM.

That macrophages exposed to LPS and IFN-γ increase iNOS expression and NO production is well known. But what is a novel clue for the understanding of NO-mediated β-cell damage is that N^G-hydroxy-L-arginine (L-NOHA), an intermediate in the biosynthesis of NO, is a potent competitive inhibitor of L-arginase I.[155,156] Indeed, substantial amounts of this metabolite are released by LPS-treated rat alveolar macrophages,[157] while inhibition of L-arginase by L-NOHA may ensure sufficient availability of L-arginine for high-output production of NO in activated cells. L-citrulline, the co-product of iNOS catalysis, and S-nitrosoglutathione, an adduct produced by the reaction of NO with GSH, are also inhibitors of L-arginase in many cell types,[156,158] including β-cells.[130] Hence, intermediates of NO synthesis, as well as NO itself, precisely coordinate a maximum of flux through iNOS in insulin-producing pancreatic cells (Figure 2). Conversely, dexamethasone and dibutyryl cAMP block both iNOS and L-arginase expression, which is paralleled by a strong decrease of NO production.[159] Additionally, macrophages treated

with LPS and IFN-γ undergo NO-dependent apoptosis, which may be prevented by L-arginase cDNA transfection.[159] In such cells, L-arginase I and II seem to play a role in avoiding NO-elicited apoptosis.

Competition between L-arginase and iNOS has also been found in activated murine macrophages incubated with another L-arginase inhibitor, nor-L-NOHA.[138] Contrarily, L-arginase induction by the type 2 cytokines IL-4 or IL-13 has been shown to inhibit macrophage NO synthesis due to increased L-arginine utilization by L-arginase.[160] Similar results have been obtained by using different cells types.[159,161] In β-cells, both L-arginase I, the major isoform expressed in rodent pancreas, and L-arginase II, the main human isoform, seem to reciprocally regulate iNOS-dependent NO production under physiological L-arginine concentrations,[134,162,163] which suggests that islet L-arginase may be able to compete with iNOS *in vivo*, where L-arginine ranges at non-saturating concentrations for both enzymes. This fact may be of relevance for β-cells during Th1-driven insulitis, since L-arginine concentrations are likely to be reduced at sites of inflammation due to the release of soluble L-arginase from infiltrating macrophages.[164] Corroborating this proposition is the fact that IL-1β-induction of NO synthesis in RINm5F β-like cells is accompanied by a reduced flux of L-arginine through L-arginase, an effect that appears to be mediated by L-NOHA.[130] Hence, it is likely that, following immune cell-elicited NO production via iNOS, L-NOHA inhibits islet L-arginase activity to some degree *in vivo*, which may be strongly exacerbated by the pro-inflammatory cytokine IL-1β, that inhibits L-arginase expression in β-cells.[132,133] In fact, a remarkable reduction in L-arginase expression has been recently observed during insulitis in the NOD mouse model of T1DM.[165]

Even more surprising is the fact that, as previously described for activated macrophages cultured in the presence of low extracellular L-arginine,[166] β-cells are also able to synthesize NO from glutamine, glutamate or alanine, since these cells express huge amounts of all enzymes and transporters needed to produce L-arginine intracellularly.[167,168] In the absence of exogenous L-arginine, glutamate becomes a mandatory substrate for NO synthesis via the urea cycle, through the concerted action of carbamoyl phosphate synthetase I, ornithine transcarbamoylase, argininosuccinate synthetase and argininosuccinate lyase that produce L-arginine in β-cells (Figure 2). Glutamate is also proposed to amplify glucose-induced insulin secretion in a K_{ATP} channel-independent way.[168] However, glutamate is, at the

same time, an obligatory substrate for GSH synthesis, which, in turn, enhances ATP/ADP ratios and scavenges ROS/RNS leading to insulin secretion. And so also is alanine, which may replenish the β-cell glutamate pool via an alanine aminotransferase-catalysed reaction. This explains why alanine is cytoprotective to β-cells against cytokine-induced apoptosis,[167] *i.e.* under cytokine-stimulated NO production, alanine may provide glutamate for GSH synthesis thus avoiding oxidative stress and NO-induced apoptosis.

Since, as discussed above, β-cells have poor NADPH-dependent GSSG reductase (GSRd) activity, necessary to regenerate GSH from GSSG in situations of oxidative stress, and NADPH production from the hexose monophosphate shunt is limited because β-cell glycolytic activity is committed to mitochondrial ATP production during glucose-stimulated insulin release, *de novo* GSH biosynthesis from glutamate becomes crucial for insulin release and avoidance of β-cell death. Hence, it is easy to envisage that any metabolic disequilibrium in providing L-arginine for NO-assisted insulin secretion, during secretagogue-stimulated insulin release, forces β-cell metabolism to handle glutamate in order to synthesize L-arginine and NO. This, in turn, diverts glutamate away from GSH synthesis leading to a redox imbalance.

The liver is the major supplier of glutamate to the circulation,[169] so that the liver is the main source of exogenous glutamate for β-cells. In turn, the kidney is considered to be the physiological producer of L-arginine since it is the only organ known to take up L-citrulline released from the metabolism of glutamine in the gut and release L-arginine into the blood (Figure 3A), although other tissues strongly express argininosuccinate synthetase and lyase but without any net delivery to the circulation.[170] In fasted humans, the contribution of glutamine via L-citrulline to the *de novo* synthesis of L-arginine is about 65% in neonates, where the gut is the major source of systemic L-arginine, even though some residual production in the adult gut could be accounted for by L-arginine release as well.[170] Consequently, if, by any chance, the flux through the coupled glutamine/L-arginine pathway between intestine and kidney is broken or faces any reduction, glutamate becomes the immediate mandatory precursor for NO synthesis (Figure 3B). Glutamate, however, is a unique source of GSH in β-cells, so that a disruption or hypofunctionality of gut-to-kidney glutamine/L-arginine pathway, would promptly decrease GSH synthesis thus reducing insulin release, leading to oxidative stress and β-cell death. On the other hand, glutamine which is a major

and immediate glutamate precursor, is also a primary nutrient for the maintenance of immune cell function.[171–173] Hence, we are tempted to speculate that an immune response triggered by whatever challenge in a redox-sensitive subject (in which the expression/activity of antioxidant and GSH enzymes is, at least at any moment, abnormally low) might decrease the availability of glutamine to L-arginine-dependent NO generation and glutamate-dependent GSH *de novo* synthesis in β-cells, thus imposing the synthesis of L-arginine from glutamate, which, in turn, would deplete the GSH biosynthetic pathway leading to oxidative stress in β-cells (Figure 3). Analogously, it seems likely that other situations, in which the circulating glutamine pool is severely endangered,[171,174,175] such as in undernourishment, strenuous-exercise or cancer cachexia-associated muscle loss, chronic inflammatory diseases, severe metabolic acidosis, major burns, polytrauma and bacteremia, should favour β-cell dysfunction.

Glutamine deficiency can occur during periods of critical illness. In patients with catabolic diseases, plasma and muscle glutamine levels are dramatically reduced, which correlates with the poor prognosis and high degree of protein catabolism in those patients. For instance, in patients with major burn injury, plasma glutamine concentration is lower than 50% of that in normal controls and it remains low for at least 21 days after the injury.[176] Conversely, in LPS-septic rats, a single dose of glutamine has been shown to attenuate the release of TNFα and IL-1β and to be associated with a significant decrease in mortality due to the attenuation of pro-inflammatory type 1 cytokines,[177] whereas L-arginine-enriched diet limits plasma and muscle glutamine depletion in head-injured rats.[178] Remarkably, however, predominately Th1 (but not Th2) cell responses require the presence of optimal concentrations of glutamine.[179] Since β-cell death that accompanies the onset of T1DM is an essentially Th1-elicited cytotoxicity, it is not unreasonable to suppose that the specific recruitment of Th1 cells may greatly enhance glutamine utilization leading to an L-arginine deficit in β-cells. This, in turn, diverts glutamate from GSH synthesis towards the L-arginine/iNOS system, which causes a reduction of insulin release and redox imbalance. If this is so, the question is why are Th1 cells specifically activated?

Many stressful conditions that are associated with immune system imbalances, including psychological ones, have long been associated with the onset of T1DM.[180,181] Indeed, in a recent study, it has been shown that stressful life events and psychological dysfunctions dramatically augment the likelihood of
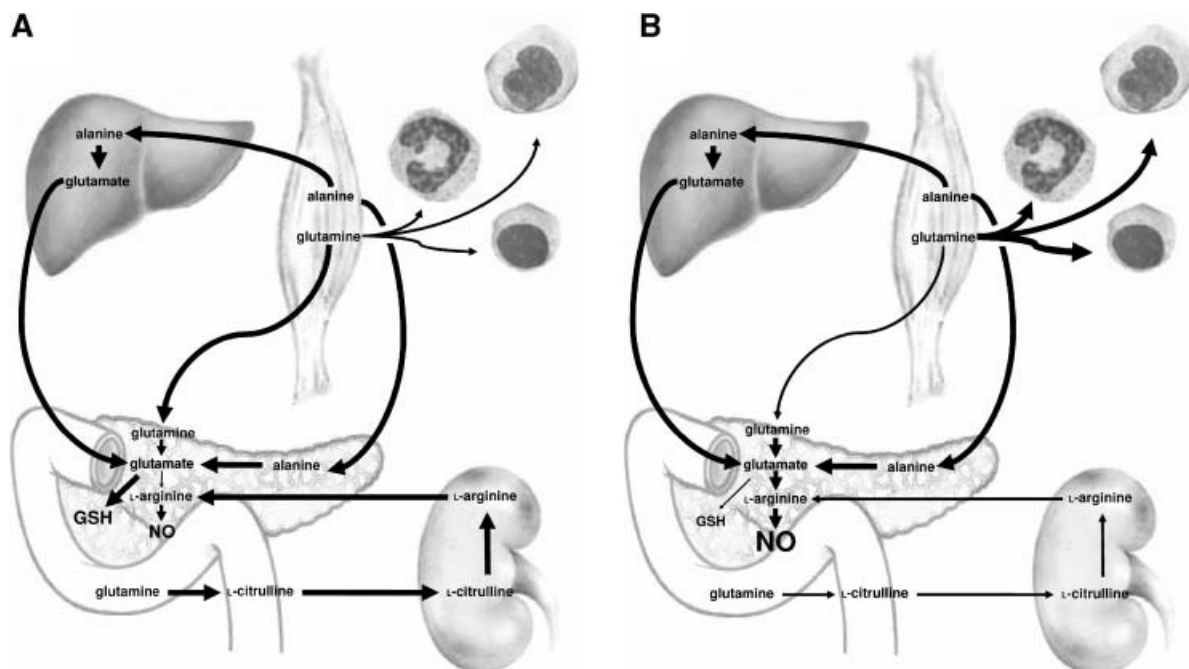
Figure 3. The L-arginine/glutamine coupling hypothesis of insulin-secreting β-cells. (A) Pancreatic islet β-cells utilize L-arginine for the biosynthesis of NO and glutamate for GSH generation during secretagogue-stimulated insulin secretion. L-arginine is provided to the pancreas by the intestine-kidney coupled conversion from glutamine, while glutamate is furnished by the liver mainly from muscle-derived alanine. Skeletal muscle-derived glutamine is also substrate for the maintenance of GSH metabolism in β-cells, but rapidly proliferating cells of the gut as well as immune cells compete with β-cell for the utilization of glutamine. Hence, any minimal reduction in the supply of L-arginine to the pancreas may shift glutamate metabolism towards the synthesis of NO instead of GSH, thus leading to oxidative stress, inhibition of insulin secretion and eventually β-cell death. This is the case of undernourishment, cancer states, trauma, sepsis, major burns and low skeletal muscle mechanical activity, where blood glutamine stores may be challenged. Metabolic acidosis, by increasing glutamine utilization by the kidney may also favour glutamine depletion unless enteral supplementation or enhanced physical activity takes place. This is also the case of psychological-stress motivated inflammatory reactions that may underlie by the activation of sympathetic-CRH–histamine system (Figure 1), which ultimately leads to a Th1-centred immune response that augments glutamine utilization. Therefore, glutamine imbalance, by virtue of deficiently supplying L-arginine to the pancreas, deviates β-cell glutamate metabolism from the synthesis of GSH to NO, leading to oxidative stress, impairment of insulin release and insulitis. This ongoing inflammation feeds forward NO metabolism, which enhances glutamine consumption thus perpetuating this cyclic condition that leads to T1DM (B). Physical exercise, on the other hand, may improve glutamine supply from the skeletal muscle and counteract Th1-mediated inflammation due to the production of type 2 cytokines, such as IL-6. Immunomodulatory action of exercise may also involve heat shock protein production and other anti-inflammatory mediators (please, see the text).

the incidence of T1DM in children and adolescents.[182] These include parents' job-related changes or lost job, severe accidents, hospitalization or death of a close friend, quarrels between parents, war, near-drowning in a pool, falling down, being an unhurt participant of an accident, conflicts with parents/teacher/neighbours, to be lost in town, physical attack, failure in competition, penalty, examination, death of pet, presence of lightning strike, loss of housing accommodation and learning problems. As a general rule, stress is considered as immunosuppressive. Surprisingly, however, a growing body of evidence strongly suggests that acute stress serves as a pro-inflammatory stimulus via the production of corticotropin-releasing hormone (CRH) by peripheral sympathetic nerve terminals.[183] CRH stimulates lymphocyte proliferation[184,185] and secretion of IL-1 and IL-2 by mononuclear cells isolated from the peripheral blood of healthy subjects.[186] Peripheral CRH exerts a pro-inflammatory effect in autoimmune diseases with a selective increase in Th1-type responses, which is probably mediated by an NF-κB-dependent pathway.[187] Additionally, it is possible that, upon a stressful situation, peripherally delivered CRH activates mast cells that secrete histamine, which acts via H1 receptors to induce local inflammation.[183] In fact, diabetes is associated with increased basal hypothalamus-pituitary-adrenal (HPA) activity and impaired stress responsiveness.[188] Therefore, psychological stress may selectively activate Th1 lymphocytes that

mediates type-1 cytokine-induced iNOS expression, exacerbated NO production and β-cell cytotoxicity. Enhanced Th1 activity, in turn, increases glutamine utilization with the consequent shift of glutamate metabolism from GSH biosynthesis towards NO production, as discussed above (Figures 2 and 3). The possibility of a peripheral CRH–receptor-mediated glutamine/L-arginine imbalance in T1DM is now under evaluation in our laboratory.

Taken together, these findings suggest that psychological stress may have a dual and cross-potentiating role in determining the onset of T1DM: an immuno-inflammatory and a metabolic one. Arguing in proof of such a hypothesis is the observation that orally administered L-arginine supplementation significantly improves patient status in a series of different pathological conditions associated with immune dysfunctions, including in pre-term neonates,[189] without increasing urea levels.[190] Curiously, intraperitoneal L-arginine injection, where the physiological coupling of glutamine/L-arginine is bypassed, does not improve diabetes in animal models. On the contrary, it seems to worsen it,[191] while oral administration of L-arginine to AL-treated rats restores blood glucose and insulin levels.[192] Oral L-arginine administration has also been shown to improve, but not completely, peripheral and hepatic insulin sensitivity in T2DM,[193] where oxidative stress[41,73,194] and NO overproduction[35,41] are also involved. If this is so, nutritional management of glutamine and/or L-arginine, enterally administered in order to allow for the physiological re-establishment of glutamine/L-arginine homeostasis,[170] may rescue β-cell redox balance in ongoing T1DM. Additionally, skeletal muscle is a major site for glutamine synthesis in the human body and contains over 90% of the whole-body glutamine pool. Quantitative studies in humans have demonstrated that, in the postabsorptive state, 60% of the amino acids released comprise alanine plus glutamine (Figure 3A).[195] Therefore, moderate physical exercise, which is known to accelerate the rate of glutamine delivery into the circulation, may be of value in protecting glutamine/L-arginine metabolic coupling between the gut and β-cells.

PHYSICAL EXERCISE, ANTI-INFLAMMATORY CYTOKINE RESPONSE AND T1DM

Regular practice of moderate-intensity physical exercise has been shown to efficiently and positively impact upon physiological imbalances caused by different pathological situations. Exercise has been prescribed as a complementary therapeutic strategy in different immunological dysfunctions.[196] A variety of studies have demonstrated that exercise induces considerable changes in the immune system function that are physiological responses to both metabolic and hormonal exercise-related alterations. Most of the exercise responses in the immune system are mediated by hormones such as adrenaline, cortisol, growth hormone (GH) and pro- and anti-inflammatory cytokines.

Exercise-induced physiological changes are dependent on exercise intensity, type and duration. For instance, cytokine production is modulated by a range of physiological stimuli that accompany exercise, such as stress hormones, energy crisis and oxidative stress.[197] In turn, exercise-induced cytokine effects depend on the type of mediator involved and the balance between pro-inflammatory cytokines (IL-1, TNF-α, IFN-α, IFN-γ, TNF-β, IL-2, IL-12 and MCP-1) and anti-inflammatory ones [IL-4, IL-10, IL-13, IL-12p40, interleukin-1 receptor antagonist (IL-1ra)].

During endurance exercise, pro-inflammatory cytokine production is downregulated and anti-inflammatory cytokines, such as IL-1ra, IL-10 and IL-6, are upregulated.[198–200] Strenuous prolonged exercise induces increases in circulating TNF-α, IL-1β and IL-6 levels. This is counterbalanced by cytokine inhibitors (IL-1ra, sTNF-r1 and sTNF-r2) and the anti-inflammatory cytokine IL-10.[201] The magnitude of the changes differs markedly depending on the cytokine being examined. For instance, plasma concentrations of IL-1 and TNF-α increase one- to two-fold, whereas IL-6 has been reported to increase over 100-fold after prolonged exercise.[201] Local inflammatory reactions may be induced during muscle cell apoptosis or necrosis by activated macrophages and by inflammatory cytokines.[202]

A large number of studies have reported increased plasma concentrations of anti-inflammatory cytokines, such as IL-1ra, IL-4 and IL-10, after various forms of exercise including brief maximal exercise,[203,204] resistance exercise,[203,205,206] downhill running,[207,208] intense eccentric cycling[209] and endurance running and cycling.[8,201,203,204,210,211] Increased production of anti-inflammatory cytokines during exercise may serve to restrict pro-inflammatory reactions to exercise-induced muscle damage[209] and may also limit the production of pro-inflammatory cytokines associated with the development of ill states.[212] Conversely, increased production of anti-inflammatory cytokines during exercise may result in

enhanced susceptibility to infections via alterations in the pro- versus anti-inflammatory cytokine balance towards a stronger anti-inflammatory response.[8]

Peake *et al.*[213] studied the effects of exercise intensity and exercise-induced muscle damage on changes in anti-inflammatory cytokines and other inflammatory mediators. They evaluated nine well-trained male runners who had completed three different exercise trials on separate occasions: (1) level treadmill running at 60% VO_2 max (moderate-intensity trial) for 60 min; (2) level treadmill running at 85% VO_2 max (high-intensity trial) for 60 min; (3) downhill treadmill running (-10% gradient) at 60% VO_2 max (downhill running trial) for 45 min. The plasma concentrations of IL-1ra, IL-12p40, MCP-1 and 70-kDa heat shock protein (hsp70) increased after all three trials. Plasma prostaglandin E_2 concentration increased after the downhill running and high-intensity trials, while plasma IL-10 concentration increased only after the high-intensity trial. IL-4 and leukotriene B4 did not alter after exercise. Plasma IL-1ra and IL-10 concentrations were higher after the high-intensity trial than after both the moderate-intensity and downhill running trials. As an explanation of their findings, the authors concluded that, following exercise up to 1-h duration, exercise intensity appears to have a greater effect on anti-inflammatory cytokine production than exercise-induced muscle damage. After exhaustive exercise with carbohydrate supplementation, cytokine levels are lower post-exercise compared to phases without carbohydrates. The lower cytokine level may indicate lower metabolic stress.[214] The above findings suggest a link between exercise-induced increase in IL-6 and glucose homeostasis, as previously hypothesized.[215–217]

Until now, IL-6 has been considered an immuno-modulatory cytokine, in the sense that it counteracts the stimulating pro-inflammatory cytokine effects. During the past few years, however, evidence has accumulated to support the proposition that IL-6 could have an active role during exercise-induced changes in immune function. In fact, the level of circulating IL-6 has been shown to increase dramatically (up to 100-fold) in response to exercise.[218–221] Exercise also increases the circulating levels of other anti-inflammatory cytokines and cytokine inhibitors, such as IL-1-receptor antagonist (IL1-ra), TNF-α receptors (TNF-α-R) and the anti-inflammatory cytokine IL-10.[201,222] In addition to this, the concentrations of the chemokines IL-8, macrophage inflammatory protein-1a (MIP-1a) and MIP-1b are elevated after strenuous exercise.[223] Most studies have reported that

exercise, *per se*, does not increase plasma levels of TNF-α, although some have shown that strenuous, prolonged exercise, such as marathon running, results in a small increase in the plasma concentration of TNF-α.[202,204,224] This long-term effect of exercise may be ascribed to the anti-inflammatory response elicited by an acute bout of exercise, which is partly mediated by muscle-derived IL-6.

Physiological concentrations of IL-6 stimulate the appearance, in the circulation, of the anti-inflammatory cytokines IL-1ra and IL-10, and inhibit the production of the pro-inflammatory cytokine TNF-α. Hence, exercise seems to downregulate pro-inflammatory cytokine production while increasing anti-inflammatory cytokine production and action, which may induce a very strong anti-inflammatory cytokine response. The main modulator of these responses is likely to be the appearance of IL-6 in the circulation. Since IL-6 strongly downregulates NF-κB activation, it is plausible to suppose that moderate exercise-induced IL-6 production may suppress NF-κB-dependent iNOS while stimulating L-arginase activity/expression with a consequent decrease in NO-dependent β-cell death upon Th1-driven β-cell assault. Therefore, besides any possible beneficial effect that moderate exercise may have on glutamine/L-arginine coupling that is responsible for the maintenance of β-cell redox homeostasis and insulin secreting capacity (see above), mild physical exercise may shut off pro-inflammatory cytokine machinery, which gives rise to an additional protection against the development of T1DM.

Exercise may also present an anti-inflammatory effect by virtue of its ability to induce the expression of the 70-kDa family of heat shock proteins (hsp70).[225] In turn, activation of the hsp70 biochemical pathway is well known to exert a powerful suppression on pro-inflammatory NF-κB activation.[54,226] Actually, the heat shock factor (HSF)/hsp70 pathway is now considered to be one of the most potent anti-inflammatory resources physiologically[227] while hsp70 expression is cytoprotective.[226] Interestingly, diabetic ketoacidosis, which may deplete circulating glutamine stores, has been shown to induce hsp70 expression.[228] On the other hand, T1DM may increase susceptibility to oxidative damage due to the impairment of hsp70-induced protection while endurance training may offset some of the adverse effects of diabetes by upregulating tissue hsp expression, possibly via transcriptionally mediated mechanisms involving HSF activation.[225] Additionally, hsp pathways are correlated with the modulation of peripheral autoimmunity,[229] whereas at

least part of the hsp70 beneficial effect may be ascribed to its ability of promoting the expression of anti-inflammatory cytokines.[230]

NO is a potential mediator of β-cell injury in T1DM whereas this nitrogen free radical has been demonstrated to be a causal component of insulin resistance via the *S*-nitrosation of cysteine-containing proteins that are critical for insulin receptor signalling, including IRS-1 and Akt/PKB.[41] Remarkably, however, NO, by promptly reacting with GSH and other sulphhydryl-containing substrates, may cause the expression of the inducible form of hsp70, which is cytoprotective and impedes TNF-α-elicited apoptosis.[231] In fact, it is well established that hsp70 expression is redox-modulated by cysteine modification of intracellular proteins and cysteine residues of HSFs,[232,233] while, under redox imbalances, hsp70 may form cysteine dimmers.[234] Hence, it is plausible to speculate that, in addition to its physiological role in insulin secretion,[40] NO may have a protective role, through the induction of hsp70, so that any deficiency in HSF/hsp70 pathways may have important implications leading to excessive pro-inflammatory cytokine-induced iNOS-dependent production of NO and β-cell toxicity. Because of this, hsp biochemical pathways have emerged as possible therapeutic targets in a number of immune dysfunctions, including diabetes,[235] while a possible defective ability of β-cells to express hsp70 in response to physiological levels of NO as a novel risk factor for T1DM deserves further studies. We are currently evaluating, in a rat model, the possibility that exercise may stimulate type-2 immune response and protect β-cells from pro-inflammatory cytokine pathways through hsp70 induction, which, ultimately, may prevent T1DM.

IL-6 AS THE MAIN MODULATOR OF IMMUNE SYSTEM CHANGES DURING EXERCISE AND ITS ASSOCIATION WITH T1DM

Physical exercise has been shown to exert an anti-inflammatory effect mediated by upregulation of Th2 cytokines and thus may lead to a protective response against the autoimmune process directed to β-cells. Among all the changes observed in anti-inflammatory cytokines profile, the appearance of IL-6 in the circulation is by far the most marked and its rise precedes that of other cytokines.[221,236,237] IL-6 is a pleiotropic and immunomodulatory cytokine, in the sense that it counterbalance pro-inflammatory cytokine actions, and has a key impact on both immunoregulation and non-immune events in most cell types and tissues outside the immune system.[238] A vast number of epidemiological, genetic, rodent and human *in vivo* and *in vitro* studies have addressed the putative role of action (or lack of action) of IL-6 in the pathogeneses underlying obesity, insulin resistance, β-cell destruction, T1DM and T2DM also. Studies on the association between IL-6 polymorphisms and human diseases, including T1DM, insulin resistance and other metabolic disorders, emphasize the importance of IL-6.[239–241] Because of this, IL-6 is a strong candidate to be the physiological mediator of β-cell protection, while dysfunctions in its production or action may be associated with the onset of T1DM.

IL-6 belongs to the IL family of cytokines, which includes IL-11, oncostatin M, leukaemia inhibitory factor, ciliary neurotrophic factor, cardiotrophin-1 and cardiotrophin-like cytokine. These cytokines are characterized by their common use of the gp130 receptor as a signalling subunit. The two IL-6 receptors, gp130 and IL-6Rα, belong to the type I cytokine receptor family, which, in addition to the above cytokines, bind leptin, growth hormone, prolactin, erythropoietin, thrombopoietin and granulocyte/macrophage-colony stimulating factors.[238] IL-6 was originally thought to be a pro-inflammatory cytokine, but this understanding was found to be simplistic[238] and, frankly, not the case. In both adaptive and innate immune system responses, IL-6 is involved in both amplification of and protection against inflammation.[238,242] Therefore, inappropriate regulation of IL-6 production and/or signalling may play either a direct protective or deleterious role in a number of immune-mediated diseases.[238,242–244]

Although IL-6 may be produced in many tissues and cell types where it plays a role in the modulation of immune system function,[245] skeletal muscle cells are a plentiful source of it physiologically.[246] Increased IL-6 gene expression and production has been reported in skeletal muscle during exercise,[247,248] muscle inflammation,[249] hypoperfusion of muscular tissues[250] and after denervation[251] or local muscle injury.[237,252] The IL-6 response to exercise has recently been reviewed.[218,221,236,237] Following exercise, basal plasma concentrations of IL-6 may increase up to 100-fold, but less dramatic increases are more frequent. Of note is the fact that exercise-induced increase of plasma IL-6 is not linear over time; repeated measurements during exercise show an accelerating increase of the IL-6 in plasma in an almost exponential manner.[216,222,253] Furthermore, the peak of IL-6 level is reached at the end of the exercise or shortly thereafter,[222,253] followed by a rapid decrease towards pre-exercise levels.

Many mechanisms have been suggested that could unravel what factor(s) may activate the production and release of IL-6 and where does it come from. Plasma IL-6 levels correlate with exercise intensity, duration, endurance capacity and the mass of muscle recruited.[218,221,236,237] Concerning the influence of cell damage on the release of IL-6 following a bout of exercise, it has been suggested that the appearance of IL-6 in the circulation is related to muscle damage,[254] but more recent studies have demonstrated clearly that muscle contractions, without any muscle damage, induce marked elevation of plasma IL-6.[209,222,255] The increase in the circulating levels of IL-6 after exercise without muscle damage is a remarkably consistent finding.[216,222,256] On the other hand, some studies have suggested a direct correlation between exercise intensity and increase of the IL-6 concentrations,[223] but it is likely that this response is related to the mass of muscle recruited and not directly to the intensity of exercise. The fact that the IL-6 response is higher during running (which involves the recruitment of more muscle groups) than in cycling provides sound evidence that the mass of muscle recruited may have a major effect on the systemic concentration of IL-6.[257,258] In fact, during a one-legged concentric knee extensor exercise model, which recruits only muscles from the upper legs, the appearance of IL-6 in the plasma is observed later and is less pronounced compared with exercises in which the recruitment of more motor units is observed.[198,253,259]

The type of muscle contraction also appears to have a great influence on the course of systemic release and appearance of IL-6. During prolonged[254] or intermittent[260] eccentric, one-legged knee extensor exercise or two-legged eccentric knee extensor exercise lasting 30 min,[254] the IL-6 level does not peak until late after the cessation of exercise. In contrast, during running, cycling or concentric knee extensor exercise, the IL-6 level peaks at the cessation of exercise before progressively declining into the recovery period.[217,222,224,261] Apart from exercise type, intensity, duration and cell damage, it has also been suggested that the exercise-induced increase in plasma IL-6 is related to the sympathetic response.[261–263] Stress-induced plasma adrenaline elevation is associated with an increase in IL-6 in the blood.[263] However, it has also been shown that when adrenaline is infused into volunteers to mimic closely the increase in plasma adrenaline during a 2.5-h running exercise, plasma-IL-6 increased only four-fold during the infusion, but 30-fold during the exercise.[264] Adrenaline thus seems to play

only a minor role in the exercise-induced increase in plasma IL-6.

To test the possibility that working muscle produces IL-6, muscle biopsies have been collected before and after exercise.[265] Before exercise, IL-6 mRNA is not detectable in the muscle, while small amounts of IL-6 protein may be detected predominantly in type I (slow oxidative) fibres in the post-exercise.[266] In response to exercise, an increase of the IL-6 mRNA content in the contracting skeletal muscle is detectable after 30 min of exercise, and up to 100-fold increases of the IL-6 mRNA content may be present at the end of the exercise bout.[267,268] The observation that intramuscular IL-6 gene expression increases in skeletal muscle in response to exercise has been confirmed in the rat model.[247] Accordingly, rats were subjected to electrically stimulated eccentric or concentric contractions of the one single leg, while the other leg remained unstimulated. Both, eccentric and concentric contractions resulted in elevated levels of IL-6 mRNA in the exercised muscle, whereas the level in the resting leg was not elevated. Therefore, it appears that the local IL-6 production is related to contracting muscle, and not to a systemic effect, because IL-6 mRNA was elevated only in the muscle from the exercising leg and not in the resting leg.

The finding of similar levels of IL-6 mRNA in both concentric and eccentric exercised muscle[224,247] supports the notion that the cytokine production cannot be as closely related to muscle damage as previously thought. In fact, although IL-6 mRNA may be detected in the resting skeletal muscle, a marked rise is observed only in the contracting one, indicating that exercise is responsible for the IL-6 gene induction.[217,224] IL-6 gene expression is not only activated in working muscle, but IL-6 protein is released in large amounts from a contracting limb and contributes markedly to the exercise-induced increase in its arterial plasma concentrations.[216] In summary, it appears that most, if not all, of the IL-6 produced during exercise originates from the contracting limbs and that the skeletal muscle cells per se are the likely source.

Different mechanisms have been found to be associated with increased synthesis and release of IL-6 during muscle contraction. These include calcium, glycogen and the activation of key transcription factors. The rate of IL-6 gene transcription is remarkably rapid after the onset of exercise, with a 10- to 20-fold increase after 30 min of exercise compared to the at rest state, which may be related to the exercise-elicited elevation in cytosolic Ca^{2+} levels.[269] The promoter region of the IL-6 gene

contains binding sites for the pro-inflammatory transcription factor NF-κB and nuclear factor IL-6 (NFIL6).[270] Additional transcription factors, such as the nuclear factor of activated T-cells (NFAT) and HSF1 and HSF2[271] may contribute to the activation of IL-6 gene transcription also. Additionally, intracellular Ca^{2+} ($[Ca^{2+}]_i$) rise activates both NFAT and NF-κB *in vitro*,[272,273] and incubation of muscle cell cultures with ionomycin, a calcium ionophore, increases IL-6 secretion in a p38 mitogen-activated protein kinase (MAPK)-dependent manner.[274] Interestingly, NF-κB and c-Jun N-terminal kinase (JNK) are selectively activated by large $Ca^{2+}]_i$ rises, whereas activation of NFAT is induced by a low sustained $[Ca^{2+}]_i$. Febbraio and Pedersen[237] have proposed that, during prolonged contractile activity that results in an increase in IL-6 mRNA in skeletal muscle,[217,257,267,275] initial IL-6 transcription occurs via a Ca^{2+}/NFAT-dependent pathway. It is likely that this should be the case because of at least two reasons. Firstly, NFAT is activated in many cells, including skeletal myocytes via the upstream activation of calcineurin (protein phosphatase 2B), which is a Ca^{2+}-dependent serine–threonine protein phosphatase cytosolically located.[276,277] Also, calcineurin is present in a 10-fold higher concentration in neuronal and muscle cells than in other cell types.[269] Secondly, when activated, calcineurin binds to and dephosphorylates NFAT, allowing it to translocate to the nucleus where it associates with other transcription factors.[269] Not only can NFAT itself lead to cytokine gene transcription, but it can also bind to the transcription factor AP-1, which can lead to cytokine gene transcription.[276,277] Although this pathway may lead to IL-6 gene transcription during sustained muscular contractions, it is possible that large Ca^{2+} transients, as seen under maximal contraction, can activate IL-6 via NF-κB and JNK. As an example, skeletal myotubes release IL-6 when exposed *in vitro* to oxidative stress, in an NF-κB-dependent way.[278] Since NF-κB is a transcription factor involved in almost all immune responses,[49] it is clear that IL-6 expression can be expected to occur under both muscle activity and injury conditions. In addition, supplementation with different antioxidants attenuates the systemic increase of IL-6 in response to exercise.[279,280] On the other hand, increased oxidative stress, as well as low glucose availability, low glycogen content, catecholamines, increased $Ca^{2+}]_i$, hyperthermia, ischemia-reperfusion are all exercise features capable of inducing hsps,[281–286] which may, in turn, activate IL-6 synthesis via HSF1 and HSF2.[271]

Depending on exercise duration and intensity, glycogen depletion and, eventually intracellular glycopenia, may take place. This may indicate that IL-6 gene transcription, and ultimately protein translation and release, may be linked to glycogen depletion. In a recent study, an elevated plasma IL-6 response has been observed when subjects exercised in a glycogen-depleted state.[287] Carbohydrate ingestion, in turn, attenuates elevations in plasma IL-6 during exercise.[261] Moreover, exercise increases the transcription rate of IL-6 gene in skeletal muscle of human subjects in a response that is dramatically enhanced under conditions in which muscle glycogen concentrations are low, suggesting that pre-exercise intramuscular glycogen content appears to be an important variable for the transcription of IL-6 gene.[267] Inasmuch as IL-6 release is linked to the regulation of glucose homeostasis during exercise, IL-6 behaves as a true sensor of intramuscular carbohydrate availability that may be communicated to the immune system.

The signalling cascade that results in IL-6 gene transcription due to altered glycogen availability is not well understood, but it is possible that low glycogen content within the muscle may simply result in an impaired reuptake of Ca^{2+} by the sarcoplasmic reticulum, leading to activation of IL-6 by the previously discussed Ca^{2+}-dependent pathway. Another possibility, however, is that low intramuscular glycogen can activate IL-6 in a Ca^{2+}-independent way. Apart from NFAT, JNK and NF-κB, IL-6 is activated by p38 MAPK which induces IL-6 in rat cardiomyocytes via the downstream activation of NF-κB.[288] It is well known that p38 MAPK increases markedly in contracting skeletal muscle.[261,289] For instance, reduction of intramuscular glycogen content prior to exercise results in increased accumulation of IL-6 mRNA and release of IL-6 within the contracting muscle,[267,274,290] which seems to be mediated by the activation of p38 MAPK[274] and adenosine monophosphate-dependent protein kinase (AMPK).[257,261,262,291–293]

The biological actions of muscle-derived IL-6 have been investigated in studies in which human recombinant IL-6 was infused in healthy volunteers to mimic closely the IL-6 concentrations observed during prolonged exercise. Most of the actions were related with glucose and fat metabolism as well as immunoregulatory mechanisms. For instance, the increased expression of IL-6 was shown to be associated with increased glucose uptake during exercise.[217] During IL-6 infusion, it is observed that a concomitant increase in liver glucose output and

increased circulating levels of free fatty acids (FFA) occurs.[294] Interestingly, Wallenius *et al.*[295] have demonstrated that IL-6-deficient mice develop mature-onset obesity and that prolonged IL-6 treatment evokes a marked decrease in body weight in the IL-6-knockout mice, but not in the wild-types. It has been further demonstrated IL-6 increases FFA concentrations as well as the rate of appearance of *de novo* synthesized FFA into the blood with no concomitant elevation of plasma triacylglycerols.[296] Furthermore, IL-6 induces both IL-1ra and IL-10,[297] which are classical anti-inflammatory cytokines known to be induced by physical exercise.[237] Moreover, IL-6, at physiological concentrations, elevates plasma cortisol levels which closely corresponds to the changes in the kinetics and concentrations of cortisol in response to exercise.[297]

Even though the effects of IL-6 on β-cells remains a matter of debate and controversies,[298] it has been found that IL-6 hinders the development of T1DM in different mouse models.[299,300] Moreover, IL-6 has proved to be effective in protecting insulin-secreting MIN6 cells and freshly isolated pancreatic islets against Th1-derived cytokine (IL-1-β, TNF-α and IFN-γ)-induced apoptosis while improving cellular viability and insulin secretion.[301] Altogether, the above propositions support an important protective effect of exercise-dependent muscle-derived IL-6 on β-cells against the development of T1DM.

CONCLUDING REMARKS AND PERSPECTIVES

The dysregulation of immune system function characteristic of Th1-elicited β-cell toxicity and impaired insulin secretion which accompany the onset of T1DM may be triggered when an individual faces a strong psychological stress that determines an enhanced glutamine utilization by Th1 lymphocytes. Since glutamine is the physiological precursor of L-arginine for the synthesis of NO, whose production in β-cells potentiates insulin secretion, the high flux through iNOS compels β-cell metabolism to divert glutamate from GSH formation towards L-arginine synthesis, via the urea cycle. The oxidative stress that takes place upon reduced intracellular GSH levels allows for the activation of NF-κB, which, in turn, positively feeds back on iNOS expression and activity, thus perpetuating the inflammatory process within β-cells where excess NO is harmful. Defective hsp70 induction in response to physiological levels of

intraislet NO may also be involved in the pathogenesis of T1DM. This possibility, however, remains to be investigated.

Physical exercise is capable of inducing a huge production and release of IL-6, which is a key anti-inflammatory mediator that suppresses NF-κB-dependent responses. Moreover, exercise-elicited activation of hsp70 biochemical pathways completely blocks NF-κB activation, impedes apoptosis and is cytoprotective due to hsp70 chaperone activity, which protects against protein denaturation. Hsp70 induction is also associated with enhanced Th2 cell activity over Th1. Metabolically, exercise may restore glutamine supply thus normalizing pancreatic production of NO from kidney-derived L-arginine, and not from glutamate which is necessary for GSH synthesis and antioxidant defence. This raises the suspicion as to whether the enormous changes in human life style, compared with that of our 3–4 million-old ancestors, could be related with our current inability in maintaining healthy β-cells. We advocate that present-day levels of physical activity and dietary patterns[302,303] seem to have changed much faster than the time needed to allow evolutionary metabolic changes. In other words, our metabolism evolved to fit a level of physical activity and availability of a variety of food supplies different from those of nowadays. Accordingly, β-cells are the 'same old ones', while the protective effect of moderate physical exercise has been effectively abolished. As a corollary, unless humans enhance their pattern of physical activity, T1DM will become more and more of a risk factor for everyone. Therefore, the notion that β-cells are solely bystanders of oxidative stress-mediated cell toxicity because their antioxidant defences fail in managing physiological stresses seems to be a clumsy misconception.

Since the glutamine/L-arginine duet may influence β-cell function and survival, the knowledge of physiologically adequate levels and fluxes of both amino acids may serve as a predictor of β-cell susceptibility to the installation of T1DM. Additionally, although the possibility of pharmacologically exploiting Th1/Th2 duality relative to L-arginine metabolism may open new avenues for T1DM therapeutics, physical exercise is still the cheapest and easiest physiological measure to avoid the onset and/or worsening of T1DM. In summary, if the prevention of T1DM is dependent on both restoration of adequate L-arginine supply to β-cells and blockage of NF-κB overstimulation, moderate physical exercise is presented as the most convenient solution for these two lacunes.

ACKNOWLEDGEMENTS

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3. ESTUDO 1: The effect of IL-6 on insulin secretion, glucose consumption, nitric oxide release, signal transduction and redox status in a clonal pancreatic β-cell line: a possible cytokine-mediated cross-talking between skeletal muscle and pancreatic β-cells?

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Short title: *IL-6 modulates pancreatic β-cell metabolism and insulin secretion*

Key words: IL-6, exercise, β-cell metabolism, insulin secretion, nitric oxide.

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Abstract:

Inflammatory cytokine release is normally correlated with disease progression and intensity in conditions such as obesity, metabolic syndrome and both type 1 and type 2 diabetes. However, given that contracting skeletal muscle produces and releases substantial amounts of the cytokine IL-6 which, metabolically, may be as important as adrenaline/noradrenaline during exercise, it is likely that this cytokine has positive effects on endocrine regulation. In this work we determined the effects of IL-6 (at an exercise related concentration of 50 pg/mL) on pancreatic β-cell metabolism, insulin secretion, nitric oxide release and redox status in a clonal rat β-cell line, BRIN BD11, over 24h of incubation. Results: After incubation in the presence of IL-6, chronic insulin secretion (in μg/mg protein/24h) was increased by 39% (994±151 *vs* 1379±162) paralleling a 36-fold rise in glucose consumption (from 0.5 to 18 μmol /mg protein) as compared to control group values. Moreover, IL-6 treatment also induced changes to the subsequent 20-min static assay which determined both basal and nutrient-stimulated insulin secretion: basal insulin secretion was increased by almost 100% in the presence of IL-6 (from 4.8±2 to 9.6±3.2 μg/mg protein/20 min) while IL-6 evoked an increase of approximately 28% in acute (20 min) nutrient-stimulated insulin secretion. Concomitantly, IL-6 enhanced both glutathione (GSH) and glutathione disulphide (GSSG) by nearly 20% without changing intracellular redox status (GSSG/GSH). Regarding signal transduction, AMPK levels were decreased by approximately 43% whereas phosphorylated AMPK levels were elevated by 89% after IL-6 exposure, suggesting that IL-6 decreases β-cell fatty acid synthesis. Additionally, IL-6 dramatically increased iNOS expression (by *ca.* 100-fold) with an accompanying 10-fold rise in nitric oxide release. It has been generally thought, for a long time, that AMPK and iNOS activation reduce insulin secretion from β-cells due to changes in lipid metabolism and generation of reactive nitrogen species respectively. However, nitric oxide has been recently shown to be indispensable for the insulin release so that our results may indicate that IL-6 exerts mainly positive effects on β-cell metabolism and insulin secretion. IL-6 may act as a communication factor between skeletal muscle cells and pancreatic β-cells during exercise, so

elevating insulin secretion to achieve optimal concentrations for glucose uptake and metabolism by contacting muscle.

Introduction

Interleukin-6 is a pleiotropic cytokine that influences metabolism in health and disease [2] and its plasma levels are acutely elevated following muscle contraction and chronically during obesity [3, 4]. With respect to its endocrine-like actions in regulating whole body metabolism, IL-6 is involved in the control of obesity, in the regulation of insulin action, and in the mobilization of energy stores during exercise [5, 6]. Systemically elevated IL-6 levels are a predictive factor for the development of type 2 diabetes in obese individuals [4, 7]. IL-6 is also a potent regulator of cell proliferation and survival, as described in liver and immune cells [2]. The two IL-6 receptors, gp130 and IL-6Ra, belong to the type I cytokine receptor family, which, in addition to IL-6, bind leptin, growth hormone, prolactin, erythropoietin, thrombopoietin and granulocyte/macrophage-colony stimulating factors [2]. Upon IL-6 binding to the IL-6 receptor (IL-6R) a complex is formed with the signal transducing transmembrane glycoprotein, gp130, and signals are transduced via activation of STAT3 and ERK signalling, regulating downstream targets such as c-myc and bcl-2 [8, 9].

The appearance of IL-6 in the circulation in response to exercise is by far the most marked and its increase in concentration precedes that of other cytokines [6, 10, 11]. Indeed, skeletal muscle is quantitatively the major physiological source of IL-6 [12] while increased IL-6 gene expression and production has been reported in skeletal muscle during exercise [13, 14].

Most of the physiologic IL-6 actions are related to glucose and fat metabolism as well as to immunoregulatory mechanisms. IL-6 increases glucose uptake during exercise [15] and augments lipolysis and fatty acid oxidation. Thus it is evident that IL-6 is a powerful lipolytic factor and it has been suggested that during exercise, the increase in arterial free fatty acid concentration is mediated, at least in part, by IL-6 released from the muscle.

Also, IL-6 exerts anti-inflammatory effects mainly by its inhibitory role on the TNF-α production and action. This finding suggests that exercise-evoked IL-6 production may mediate training-induced enhanced insulin sensitivity in humans [16, 17]. Indeed, exercise does improve insulin sensitivity in liver, muscle, and fat cells [18] and a single exercise session has been shown to increase insulin sensitivity for 16 h longer [19].

The mechanism(s) by which IL-6 exerts its diverse effects within target cells has been suggested to include AMP-activated protein kinase (AMPK). There is now accumulating evidence for a direct correlation between IL-6 concentration and AMPK activity [20, 21]. AMPK activation stimulates fatty acid oxidation and increases glucose uptake [22]. Moreover, IL-6 was shown to enhance AMPK activity in both skeletal muscle and adipose tissue [23] and, more recently, to enhance glucose uptake in skeletal muscle [24]. Furthermore, IL-6 actions have also been attributed to the activation of the suppressor of cytokine signaling-3 (SOCS3) expression in the liver and skeletal muscle cells, which results in significant changes in glucose metabolism [24, 25].

It has been known since the 1970s that insulin levels could increase immediately upon cessation of prolonged or maximal intensity exercise [26, 27]. The mechanism to explain the increase in insulin secretion after exercise has been attributed, at least in part, by the abrupt abatement of catecholamine release on termination of exercise [28]. On the other hand, it is possible that factors other than the decreasing level of adrenaline are responsible for the large increase in serum insulin after exercise so aiding the body during the recovery phase. The role of IL-6 in the pancreatic islet is still unclear. However, given that (a) elevated IL-6 levels are an independent predictor of type 2 diabetes, (b) in vivo, high-fat (HF) diet feeding increases systemic IL-6 levels, which are necessary for expansion of pancreatic α-cell mass and maintenance of fasting circulating glucagon, and (c) IL-6 has been described to regulate α-cell function in the pancreatic islet [29], we decided to investigate the pancreatic β-cell as a possible additional target of IL-6 actions.

We hypothesized that pancreatic β-cell exposure to an exercise associated concentration of IL-6 (50pg/mL) would initiate changes in the metabolism that would favour both cell defence and insulin secretion. We also

believed that IL-6, through the activation of key metabolic enzymes such as AMPK and, also, by its anti-inflammatory action, is able to induce beneficial metabolic changes in β cells. We argue that IL-6 could be responsible for exercise related communication between skeletal muscle and pancreatic β-cells during and after exercise.

Materials and Methods

Culture of BRIN-BD11 pancreatic β-cells and measurement of Insulin secretion

Clonal rat insulin-secreting BRIN-BD11 cells were maintained in RPMI-1640 tissue culture medium supplemented with 10% (v/v) foetal calf serum (FCS), 0.1% (v/v) antibiotics (100 U/mL penicillin and 0.1 mg/mL streptomycin) and 11.1mmol/l D-glucose, pH 7.4. The clonal β-cell line BRIN-BD11 was chosen for this work as its metabolic, signalling, insulin secretory and cell viability responses to glucose, amino acids, as well as other stimuli, have been well characterized [30-32]. The origin and the characteristics of BRIN-BD11 cells are described elsewhere [30]. The cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air using a Forma Scientific incubator (Marietta, OH, USA). The cells were cultured in 50–70 mL RPMI-1640 tissue culture medium in T175 sterile tissue culture flasks. Cells were subsequently seeded into six-well plates (0.75×10^5 cells per well) and allowed to adhere overnight. Cells were then washed with PBS after which they were incubated in fresh media, containing 11.1 mM D-glucose, 2 mM L-glutamine, in the absence or presence of IL-6 (50 pg/mL) added. After 24-h incubation, an aliquot of the media was removed and centrifuged at 200 *g* for 5 min and used for quantization of insulin and, at 16000 *g* for 10 minutes for the determination of metabolites (D-glucose consumption, urea and nitrites). After the 24-h incubation, the cells were acutely stimulated for 40 min in the presence of 1.1mmol/l glucose followed by 20 min in the presence of 16.7 mM glucose and 10 mM alanine (a stimulus that results in a robust and reproducible secretory response in normal conditions [33]), when an aliquot of the incubation medium

was removed, centrifuged at 200 *g* for 5 min to be analyzed for acute insulin secretion using the Mercodia UltrasenstiveRat Insulin ELISA kit.

Enzymatic determination of metabolites

Urea production

Urea was measured on 50-µl samples using the 96-well QuantiChromTM Urea Assay Kit (DIUR- 500) procedure at 520 nm, according to manufacturer's instructions, using an automatic enzyme-linked immunosorbent assay plate reader. The rate of urea production was expressed as μmol/mg of protein per time of incubation.

Nitrite Production

Nitric oxide (NO) concentrations in the culture media, estimated as nitrite production, were determined using the Griess Reagent System (Promega Medical Supply Co.). Absorbance at 530 nm was measured using an automatic enzyme-linked immunosorbent assay plate reader. The rate of nitrite production was expressed as μmol/mg protein per time of incubation.

Glucose consumption and glutamate production

A glucose and glutamate concentrations were measured in the incubation medium using an YSI 7100 amino acid analyser (YSI Incorporated). Briefly, an enzyme specific for the substrate of interest was immobilized between two membrane layers (polycarbonate and cellulose acetate). The substrate is oxidized as it enters the enzyme layer, producing hydrogen peroxide, which passes through cellulose acetate to a platinum electrode where the [hydrogen peroxide is oxidized](). The resulting current is proportional to the concentration of the substrate.

Cell viability measurement

Cell viability was determined using the cell proliferation reagent WST-1, a tetrazolium salt that is cleaved by mitochondrial dehydrogenases in viable cells. Briefly, 100 μL of cell suspension (containing 2×10^4 cells/well) were plated in

96-well plates. After a 24-hour reattachment period in culture, the cells were treated with IL-6 (50 pg/mL) for further 24 h. At the end of each experiment, the cell proliferation reagent WST-1 (10μL) was added to each well and the cells were incubated at 37°C for 0.5 to 1.5 h. Absorbance at 450 nm was then measured using an automatic enzyme-linked immunosorbent assay plate reader.

Protein determination

Cellular protein was determined using a BCA protein assay kit (Pierce, Rockford, IL, USA kit no. 23225), which utilizes a modification of the biuret reaction.

Preparation of protein extracts from BRIN-BD11 cells

BRIN-BD11 insulin-secreting cells were lysed using RIPA lysis buffer (supplied at 10× concentration; MSC, Dublin, Ireland) containing protease inhibitors. One hundred and fifty μL of 1× RIPA lysis buffer were added to the cells. Cell lysates were transferred to fresh ice-cold Eppendorf tubes and were then placed on a shaker at 4°C for 15 min. Afterwards, cells were centrifuged at 14,000 g for 15 min at 4°C. The supernatant fraction was placed in a fresh tube and stored at −20°C.

Western blot analysis

Cells were seeded into 6-well plates (1.5 x10^6 cells per well) and allowed to adhere overnight. Cells were then washed with PBS after which they were incubated in fresh media, containing 11.1m M D-glucose, 2 mM L-glutamine, in the absence or presence of IL-6 (50 pg/mL) for 24 h. After the incubation, using RIPA lysis buffer (Upstate Biochemicals, Lake Placid, NY, USA), equal BRIN-BD11 cell protein extracts were prepared. Samples were subsequently subjected to 10% SDS-PAGE and electrophoretically transferred onto a nitrocellulose sheet. The sheet was blocked in 5% milk protein and incubated with polyclonal anti-iNOS, AMPK-P or AMPK antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After incubation with appropriate secondary horseradish peroxidase (HRP)-labelled antibodies, the membranes were washed and probed with Supersignal West Pico chemiluminescent

substrate (Pierce). Results are expressed as mean ± S.D. using GAPDH as an expression control. P≤0.05.

Measurement of glutathione (GSH) and glutathione disulfide (GSSG) content

Clonal insulin-secreting BRIN-BD11 cells were seeded into six-well plates (2×10^6 cells per well) and allowed to adhere overnight. Cells were then washed with PBS after which they were incubated in fresh media, containing 11.1 mM D-glucose, 2 mM L-glutamine, in the absence or presence of IL-6 (50 pg/mL) added and prepared for the measurement of GSH and GSSG contents. For GSH and GSSG measurements, cells were rinsed twice with PBS and disrupted in 200 μL of 5% (w/v) metaphosphoric acid (MPA) on ice. After centrifugation (16,000 x g, 5 min at room temperature), cell lysates were spectrophotometrically (415 nm) assayed on a microplate reader (Molecular Devices SpectraMax Plus 384 - temperature-controlled reader) by modification of the 5,5'-dithiobis(2-nitrobenzoic acid) [DTNB, Sigma]/GSSG reductase (Sigma) recycling method, using the N-ethylmaleimide (NEM, Fluka) conjugating technique for GSSG sample preparation [34]. Samples (10 μL), for both GSH and GSSG determinations, were assayed in 105-mL final volume in 96-well polystyrene plates (Corning) at 37°C in the presence of 10 mM DTNB, 0.17 mM β-NADPH (Sigma, dissolved in 0.5% (w/v) NaHCO$_3$ as a stabilising agent) and 0.5 U/mL GSSG reductase (EC 1.6.4.2, Sigma). Results were expressed in μmol per mg of protein.

Statistical analysis

The results are presented as mean±S.D. Groups of data were compared using unpaired Student's t-test or ANOVA where appropriate. Differences were considered significant at a P values of < 0.05.

Results

IL-6 increases insulin secretion and glucose consumption in tissue culture (24 h) and subsequent acute (20 min) basal and nutrient stimulated insulin secretion

After 24-h incubation with IL-6 (50 pg/mL), BRIN-BD11 cells increased insulin secreted into the tissue culture medium by over 38% (Fig. 1A) which was paralleled by a dramatic enhancement of glucose consumption (*ca.* 36-fold compared to control groups; Fig. 1B), indicating enhanced metabolic stimulus-secretion coupling. IL-6 treatment not only increased insulin secretion over 24 h but also induced changes in subsequent acute (20 min) basal and nutrient stimulated levels. Additional 20-min incubations in the presence of IL-6 were found to increase basal insulin secretion by 100% (Fig. 1C). Moreover, when cells were submitted to a robust nutrient challenge (incubation in the presence of 16.7 mM glucose plus 10 mM L-alanine for 20 min), IL-6-treated cells demonstrated a further 27.6%-increase in insulin release, above the nutrient-stimulated level (Fig. 1C).
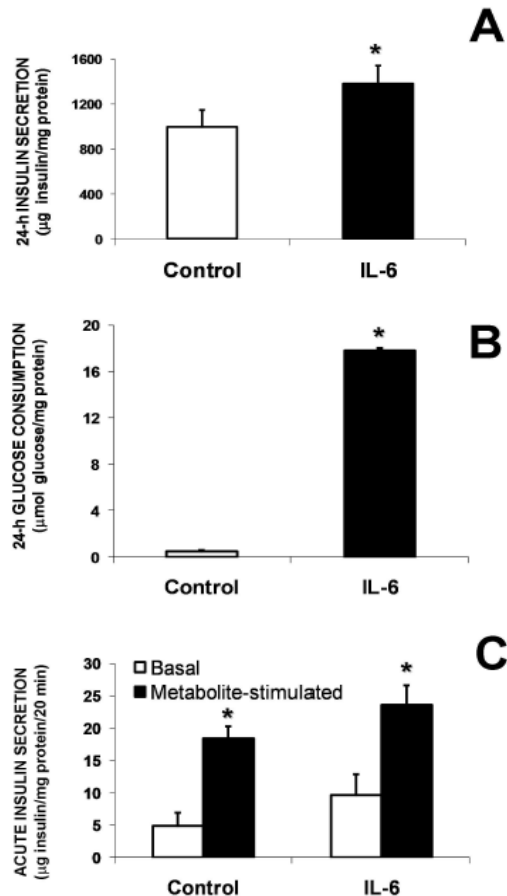
Figure 1. Insulin secretion and glucose consumption. Clonal insulin-secreting BRIN-BD11 cells were seeded into 24-well plates (0.75x105 cells/well) and allowed to adhere overnight. Cells were then washed with PBS and cultured in fresh media, containing 11.1mM D-glucose and 2mM L-glutamine, in the absence or presence of IL-6 (50 pg/mL). After 24 h, an aliquot of the media was removed and used for quantization of insulin (A) and glucose consumption (B). After the first 24-h period, cells were washed and 'rested' for 40 min in the presence of 1.1mmol/l glucose in KRB pH 7.4 (and absence of IL-6) followed by an acute stimulation period of 20 min in the presence of either 16.7mM glucose and 10mM alanine (a stimulus that results in a robust and reproducible secretory response in normal conditions [4]) or basal 1.1mM glucose in KRB pH 7.4, when an aliquot of the incubation medium was assessed for insulin as described in the Methods section. Data are the means ± S.D. of at least 3 separate determinations repeated at least two times.

IL-6 promotes AMPK phosphorylation but reduces enzyme expression

AMPK is a serine/threonine protein kinase, which serves as an energy sensor in all eukaryotic cell types. It responds to a decreased ATP/AMP ratio by enhancing processes that generate ATP and inhibiting others that consume ATP but are not acutely necessary for survival. Nevertheless, for full activation of AMPK, the enzyme must be phosphorylated. In our model, treatment with IL-6 (50 pg/mL) for 24 h induced an 89%-increase in the level of phosporylated AMPK (AMPK-P) protein whereas the expression and the inactive unphosphorylated form was decreased by 43% (Fig. 2).
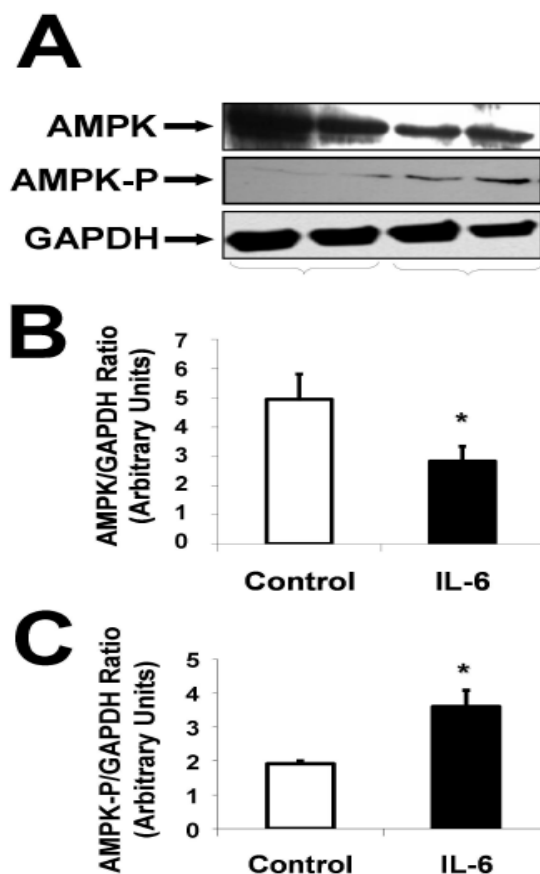
Figure 2. Determination of AMPK and AMPK-P expression. BRIN-BD11 cells were seeded into 6-well plates (1.5x106 cells/well) and allowed to adhere overnight and cultured for 24 h, as described in the legend of Fig. 1. Afterwards, equal amounts of BRIN-BD11 cell protein extracts were prepared, electrophoresed (10% SDSPAGE) and transferred onto a nitrocellulose sheet. AMPK and its phosphorylated form were then probed with specific antibodies and visualized after horseradish peroxidase/chemiluminescent reaction. Results are presented in arbitrary units relative to GAPDH expression as the means ± S.D. of 3 individual experimental preparations each in duplicate. A representative gel in duplicate is shown. *P<0.05.

IL-6 increases iNOS expression with concomitant elevation in the production of nitric oxide and decreased levels of urea production

The addition of IL-6 to culture medium caused a dramatic increased in the expression of iNOS in BRIN BD11 β-cells, from barely detectable values up to about 110-fold compared with control cells (Fig. 3). Indeed, IL-6 incubation

markedly enhanced nitric oxide production, as measured by nitrite release (from 0.34 ± 0.12 to 3.59 ± 0.86 μmol NO/mg protein/24h; Fig. 3C). On the other hand, IL-6 decreased the level of urea production by about 20% (Fig. 3D), thus suggesting a deviation of L-arginine metabolism to favour NO secretion during IL-6-mediated rise in insulin secretion.
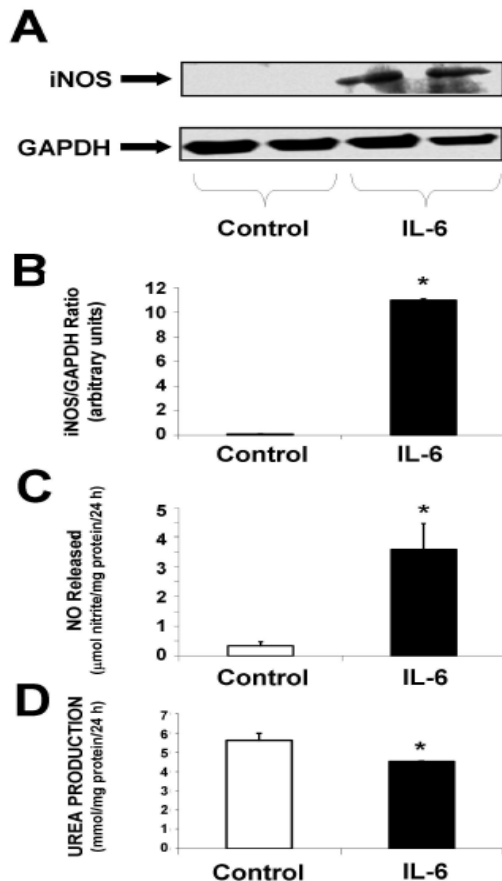
Figure 3. Determination of iNOS expression and NO metabolism. BRIN-BD11 cells (1.5×10^6 per well) were prepared, cultivated and electrophoretically analysed as described in the legend of Fig. 2 to be assessed for iNOS expression. Gel results are presented in arbitrary units relative to GAPDH expression. A representative gel in duplicate is given (A and B). NO production was inferred from the nitrite production to the 24-h incubation medium by the Griess reaction (C) whereas urea levels were measured by using QuantiChromTM Urea Assay Kit (D). Data are the means $\pm$ S.D. of 3 individual experimental preparations each in duplicate. *P<0.05.

The effects of IL-6 on cell viability, glutathione metabolism and redox status

No changes occurred in cell viability with respect to any treatment described in this study after 24 h of incubation. The assessment of glutathione metabolism and the cell redox status, given by the [GSSG]/[GSH] ratio [34], showed that IL-6 induces rise in the content of reduced GSH by approx 21 % (Fig. 4A) which was accompanied by an 21-% increment in the oxidized form (GSSG) (Fig. 4B), without any change in the redox status however (Fig. 4C).

Production of glutamate, a necessary amino acid for the GSH synthesis, was also augmented (*ca.* 36%) in the presence of IL-6 (Fig. 4).
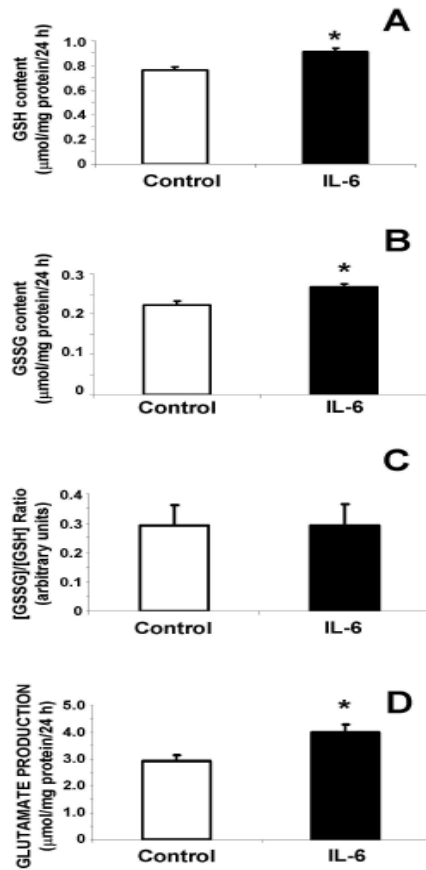
Figure 4. Glutathione metabolism and redox state. BRIN-BD11 cells were seeded into 6-well plates (2x106 per well) and treated as described in the legends of the previous figures. After 24-h incubation, cells were prepared for the spectrophotometric measurement of GSH (A) and GSSG (B) contents by the DTNB/GSSG reductase recycling method, using the N-ethylmaleimide (NEM) conjugating technique for GSSG sample preparation as described in the Methods section. Intracellular redox status, given by the [GSSG]/[GSH] ratio was also calculated (C). Glutamate released into the culture medium was also assessed (D). Results are expressed in µmoles of each metabolite per mg of cellular protein in terms of the means ± S.D. of at least 3 individual preparations. *P<0.05.

Discussion

Previous studies have reported that the exposure of pancreatic β-cells to a sub-lethal concentration of pro-inflammatory cytokines (IFN-γ, TNF-α, IL-1β) appears to shift β-cell metabolism away from a key role in stimulus–secretion coupling towards a catabolic state, which may be related to cell defence [35]. The results reported herein indicate that the cytokine IL-6, at exercise-related doses, increases insulin secretion in BRIN-BD11 cells after 24 h in culture and in response to a further acute (20 min) nutrient stimulus (Fig.1). These findings are similar to the positive effects of IL-6 on glucagon secretion and α-cell mass recently reported elsewhere [29]. IL-6 has also been found to protect MIN6 cells

(insulin secreting cell line) and isolated primary islets against the cytotoxic and insulin-inhibiting effects of IL-1β, TNFα and IFNγ [36].
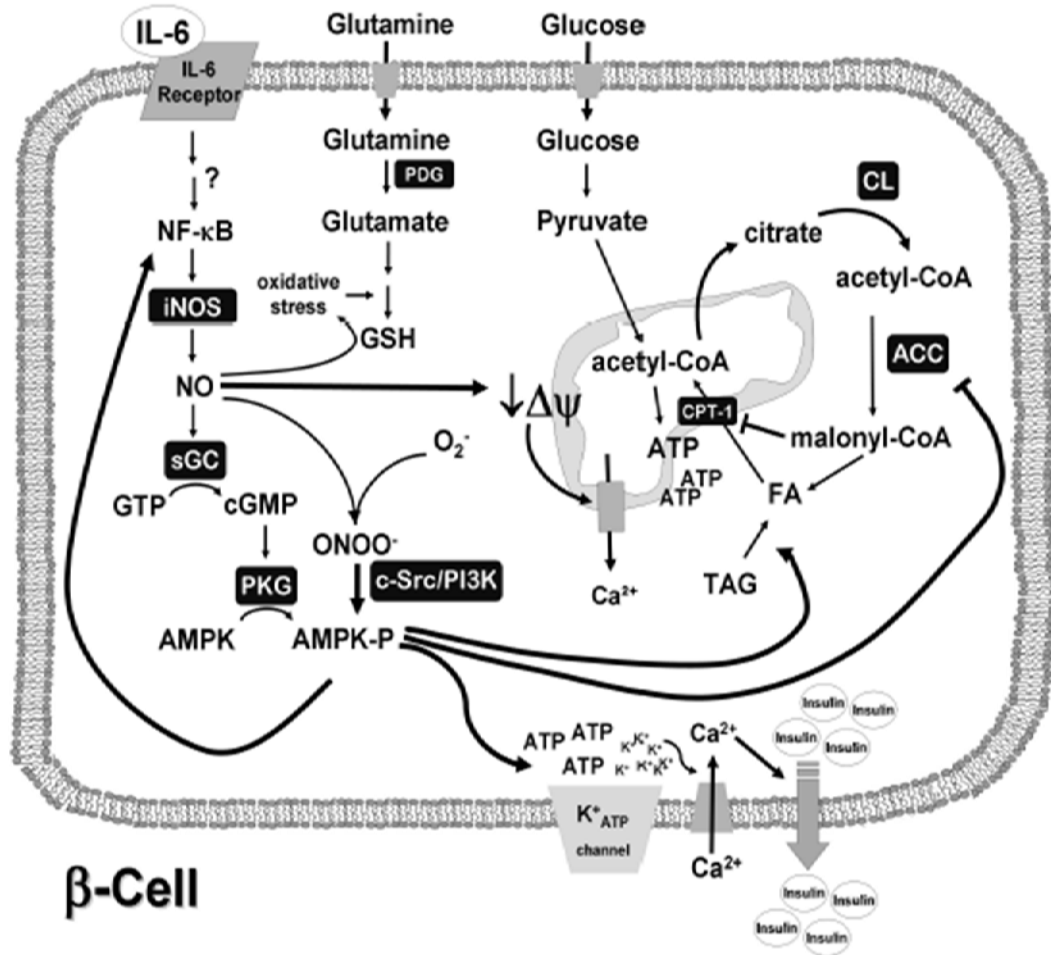
Figure 5. Proposed mechanism for IL-6-induced increase in insulin secretion through iNOS/AMPK interplay. The binding of IL-6 to its receptor located at β-cell membrane causes increment in nitric oxide synthase-2 (iNOS) expression, leading to the production of nitric oxide (NO) which, in turn, activates soluble guanilyl cyclase (sGC) with consequent production cGMP and protein kinase G (PKG) activation. The resulting phosphorylation of AMPK triggers the inhibition of acetyl-CoA carboxylase (ACC) blunting fatty acid (FA) synthesis while stimulating fatty acid degradation because the reduction in malonyl-CoA concentrations liberates carnitine palmitoyltransferase 1 (CPT1), the rate-limiting step of mitochondrial fatty acid oxidation. On the other hand, elevated fatty acid oxidation increases the ATP/ADP ratio thus inducing closure of KATP channels, membrane depolarization, calcium entrance and then insulin secretion. PKG activation is also able to activate triacylglycerol (TAG) lipases resulting into fatty acid oxidation, ATP production and insulin release. Furthermore, PKG can directly bind to β-cell KATP channels inducing their closure and eventually to insulin release. NO itself induces, by binding to cytochrome c and cytochrome oxidase, inhibition of electron transport/mitochondrial respiratory chain activity which, in turn, leads to mitochondrial depolarization and calcium release from intracellular compartments that culminates in insulin release. Peroxynitrite (ONOO-), generated from the spontaneous reaction of NO with NADPH oxidase-derived superoxide anion (O2-), activates AMPK through a c-Src-mediated and phosphatidylinositol 3-kinase (PI3K)-dependent pathway. Finally, NO, by virtue of its ability of reacting with glutathione (GSH) and of producing reactive oxygen and nitrogen species (ROS/RNS), can induce changes in the redox status leading to the production of more GSH molecules for the maintenance of the redox status. This causes also an enhancement of glutamine consumption for the generation of glutamate, which is necessary for GSH synthesis, via phosphate-dependent glutaminase (PDG). For details and references, please see the text. CL, ATP-citrate lyase; ψ, membrane potential difference.

The enhancement of ATP/ADP ratio due to increased glycolysis, glucose oxidation and metabolism is directly linked to insulin secretion [37]. Hence, activation of lipid and glucose oxidation will promote, in most circumstances, insulin secretion. In order to explain the mechanisms as to how IL-6 induced a so marked raise in insulin secretion, we measured the expression AMPK, which regulates energy consumption in β-cells. As shown in Fig. 2, IL-6 enhanced the levels of the AMPK-P (phosphorylated state, active form of the enzyme) while that of the dephosphorylated (inactive) form was reduced (perhaps, due to an IL-6-mediated ATP-sparing reduction in protein synthesis), suggesting that IL-6 may increase fatty acid oxidation and energy production due to a consequent decrease in the concentration of malonyl CoA, an allosteric inhibitor of carnitine palmitoyltransferase 1 (CPT-1), the rate-limiting step for long-chain fatty acyl-CoA transfer into mitochondria and further oxidation (Fig. 5). Therefore, IL-6-induced augmentation of fatty acid oxidation, which leads to a rise in ATP/ADP ratio, may be, finally, a mechanism by which insulin secretion was stimulated.

It has long been recognized that L-arginine, the immediate precursor of NO, is one of the most potent secretagogues for β-cell insulin release [38], while L-arginine deficiency is associated with insulinopenia and failure to secrete insulin in response to glucose [39]. Hence, although NO may be cytotoxic for β-cells at high concentrations [for review, see [1], L-arginine-derived NO might be involved in the secretagogue actions of L-arginine, and this seems actually to be the case. NO has been incontestably shown to be a physiological regulator of insulin secretion in β-cells, in an elegant experimental protocol designed by the Prof. Anne Marie Salapatek's group in Canada, in a seminal paper [40] that deserves deep and meticulous reading. They have also reported that endogenous NO production can be stimulated by glucose, and that this stimulation can be blocked by NOS inhibition, whereas scavenging of NO specifically blocks insulin release stimulated by physiological intracellular concentrations of NO-donors (2 mM), but has no effect on the release stimulated by elevated K^+. It has also been found that NO donation does not elicit a β-cell intracellular Ca^{2+} ($[Ca^{2+}]_i$) response by itself, but was able to potentiate a glucose-induced $[Ca^{2+}]_i$ response. Since NO is a powerful heme-reactant, it partially inhibits the mitochondrial respiratory chain by binding to

cytochrome *c* and/or cytochrome oxidase. As a consequence, the mitochondrial membrane potential decreases and Ca^{2+} leaves the mitochondria. This is followed by restoration of the mitochondrial membrane potential and Ca^{2+} reuptake by mitochondria. Therefore, overproduction of NO under massive inflammatory stimuli (*e.g.* in a high TNF-α, INF-γ, IL-1β environment) does merit worry, not NO production itself. As previously argued [40], the precise level of NO is crucial in determining its resultant effect, with low levels being involved in physiological signalling and higher levels becoming cytotoxic [41, 42].

Taking into account the above propositions, the massive iNOS expression and resulting NO production induced by IL-6 in BRIN-BD11 insulin-secreting cells (Fig. 3) may be due to the activation of a pre-existing physiological route devoted to NO-facilitation of insulin release in response to post-stress (*e.g.* exercise) levels of IL-6 (Fig. 5). Corroborating this possibility, the concerted reduction of urea production in face of an augmented NO release is similar to the competition observed between iNOS and L-arginase for the L-arginine catalysis in stimulated macrophages [43]. Moreover, NO may stimulate insulin secretion by directly acting on β-cell K_{ATP} channels [44].

Besides possible NO-dependent effects on β-cell electrical activity, this gas can also induce metabolic changes in β-cells by increasing the rates of glucose transport and oxidation, augmenting lipolysis and evoking mitochondrial biogenesis, effects that are mediated by multiple cGMP-dependent pathways [45]. Therefore, the conspicuous stimulatory effect of IL-6 on glucose utilization and secretion reported herein (Fig. 1) support the concept of an IL-6-related interference on mitochondrial activity and stimulus-secretion coupling [40].

In addition to that described above, a growing body of evidence suggests that IL-6 also operates as a signal for the metabolic communication between L-arginine and energy metabolism, inasmuch as NO regulates AMPK activity. Accordingly, it has recently been demonstrated that NO-donors may activate AMPK-dependent responses via the generation of cGMP, whereas inhibition of either AMPK or NO synthase abolishes these effects, indicating that NO may actually regulate AMPK, possibly via control of cellular activity of AMPK and/or AMPK phosphatases [46]. Also, a novel AMP/ATP ratio-independent pathway

for the activation of AMPK has recently been demonstrated in animal cells in which peroxynitrite (ONOO⁻), generated at non-toxic concentrations, from the spontaneous reaction of NO (•NO) with superoxide anion (O2•⁻), activates AMPK through a c-Src-mediated and phosphatidylinositol 3-kinase (PI3K)-dependent pathway [47]. Conversely, there is a number of NF-κB-linked pro-inflammatory pathways that involve iNOS expression and NO production which are dependent on AMPK activation, while pharmacological ablation of AMPK activation blocks inflammatory responses [48, 49], so that NO and AMPK downstream cascades seem to be related to each other reciprocally. Corroborates with the above propositions the finding that the oral hypoglycaemic agent metformin impairs NF-κB-dependent activation of TNF-α production through a PI3K-dependent AMPK phosphorylation in endothelial cells [48]. Altogether, these observations are in line with the results presented here (Fig. 2 and 3) and suggest that IL-6-inducing situations (*e.g.* exercise) may signalize endocrine cells via NO-AMPK coupling downstream pathways (Fig. 5).

NO is a strong redox reactant being able to oxidize the sulphidryl moiety of GSH and cysteine-containing proteins and transcription factors. Hence, as observed for other electrophiles [50], IL-6-induced NO production is capable of enhancing GSH production, in an extent that is proportional to GSH consumption (GSSG formation), via the enhanced transcription of γ-glutamylcysteine synthetase (γ-GCS, also known as glutamate cysteine ligase), the rate limiting enzyme of *de novo* GSH biosynthesis, through the activation of Nrf2 transcription factor because cytosolic Nrf2 inhibiting protein (Keap1) has a critical cysteine moiety which is redox-sensitive [50]. IL-6-stimulated increase in the production of glutamate (a necessary amino acid for the GSH synthesis), as shown in Fig. 4D, seems to be also a strategy for the maintenance of GSH-centred redox status and suggests IL-6-induced glutamine hydrolysis, since increase in glutamate influx is unlikely as glutamate concentration in the culture medium (0.14 mM) is much lower than that of glutamine (2 mM) making the latter a preferred amino acid to feed γ-GCS with glutamate.

It has been reported that the expression of IL-6 receptor in human and rodent isolated islets is associated with the α-cells and that the effect of IL-6 (200 ng/mL) was to increase glucagon secretion and α-cell mass expansion

without any influence on β-cell or insulin release [35]. Conversely, IL-6 concentration used in the present study is much lower (50 pg/mL) being likely that insulin secretion by β-cells, which is extremely susceptible to oxidative or nitrosative stress, had been temporarily blunted to favour cellular defences when clonal BRIN-BD11 β-cells were incubated with sub-lethal doses of pro-inflammatory cytokine cocktail [35].
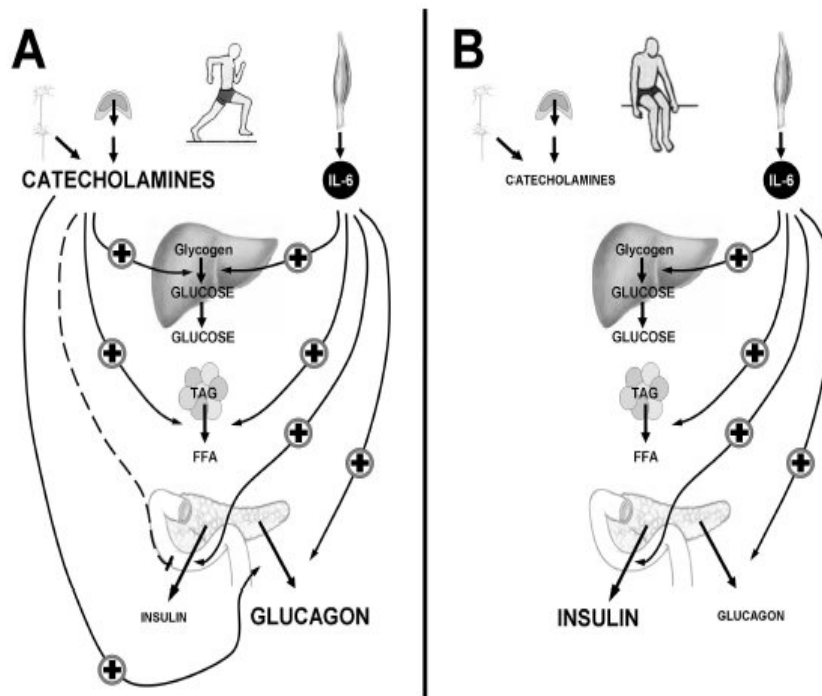
Figure 6. The hypothesis of skeletal muscle/β-cell cross-talk. (A) During exercise, skeletal muscle cells release considerable amounts of IL-6 into the blood while adrenal gland- and sympathetic nervous system-derived catecholamines also reaches bloodstream and target organs. Both IL-6 and catecholamines exert adipose tissue centred lipolysis and hepatic glycogenolysis for the maintenance of energy balance during exercise demand. Moreover, besides adrenergic-mediated depression in insulin release during exercise, IL-6 and catecholamines induce liberation of glucagon from pancreatic α-cells in order to support continuation of the exercise. (B) After exercise, a metabolic adaptation and a recovery phase is initiated in which a sharp reduction of catecholamine levels abolish the inhibition on insulin secretion. During the post-exercise period, besides its glycogenic and energy-sparing features, IL-6 exerts long-lasting effects by stimulating insulin secretion and inhibiting TNFα production by different tissues and cells, a scenario that results in adequate glucose supply for the circulation while preventing insulin-induced hyperglycaemia.

Taken together, our results and the above considerations led us to hypothesize a physiological role for IL-6 as a mechanism of cross-talking between skeletal muscle and β-cells during and after exercise (Fig. 6). The energy required for the maintenance of muscle contraction during moderate exercise is provided mainly from muscle glycogen, plasma fatty acids and glucose, but glycaemia must be maintained during exercise for CNS function, so that besides catecholamine classical effects on hepatic and muscular

glycogenolysis, adipose-tissue directed lipolysis and α-cell targeted glucagon secretion, skeletal muscle-derived IL-6 emerges as an additional glycaemia-protecting factor inasmuch as IL-6 also boosts glycogenolysis, lipolysis and glucagon secretion. IL-6 peaks as the exercise load reaches its maximum and, soon after physical exercise ceases, there is an abrupt fall in catecholamine levels while IL-6 plasma concentrations decrease much slowly. Hence, during post-exercise recovery phase, IL-6 compensate for the absence of catecholamines by stimulating both energy-furnishing pathways (*e.g.* glucose availability) and insulin secretion to avoid hyperglycaemia. IL-6 presents also metabolic effects that are not shared with catecholamines, such as inhibition of exercise-induced TNFα release by different cell types, which reduces TNFα-induced insulin resistance [51], thus favouring glucose utilisation throughout and leads the body to an anabolic compensatory state after moderate physical activity.

The fact that IL-6 released during and after exercise is capable of promoting both insulin and glucagon secretion while increasing insulin sensitivity is extremely important for the understanding of the molecular bases of aerobic exercise-evoked improvement of glycaemic control in diabetes. These possibilities are currently under investigation in our laboratory.

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4. ESTUDO 2: Does the L-arginine availability determine the beta-cells fate during the insulitis?

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Abstract:

OBJECTIVE - Since nitric oxide (NO) assists in insulin secretion, we investigated whether L-arginine manipulation could determine a shift of intraislet glutamate metabolism from the synthesis of glutathione (GSH) to that of L-arginine, leading to a redox imbalance that activates NO-mediated cytotoxicity.

RESEARCH DESIGN AND METHODS - Clonal BRIN-BD11 β-cell line were cultured for 24 h in at different L-arginine concentrations in the presence or absence of a sub-lethal pro-inflammatory cytokine cocktail (IL-1β, TNFα, IFNγ) and assessed for the production of GSH, GSH disulfide (GSSG), glutamate, NO, urea, lactate and insulin, and for the consumption of glutamine and glucose. The expressions of NO synthase (NOS-2), AMPK and the redox-protecting heme-oxygenases (HO-1 and HO-3) were also evaluated.

RESULTS - Increases in L-arginine levels dramatically raised glutamate and GSH synthesis with a concomitant reduction of GSSG intracellular concentrations and no parallel consumption of glutamine. L-Arginine stimulated GSH metabolism regardless the presence of inflammatory cytokines. Also, glucose consumption was increased by up to 80-fold during enhanced L-arginine exposure, even in the presence of the cytokine cocktail. L-Arginine stimulated AMPK phosphorylation and HO-1 expression. Insulin secretion, however, did not accompanied glucose consumption.

CONCLUSIONS - The results suggest that L-arginine stimulates β-cell anti-oxidant responses, glucose and fatty acid metabolism at the expense of stimulus-secretion coupling in conditions of inflammatory stress.

INTRODUCTION

The adverse immune response characteristic of type 1 diabetes is induced and promoted by the interaction of genetic and environmental factors which culminate with an autoimmune response directed towards specific β-cell autoantigens, insulitis and β-cell death (please, see reference (1) for review). Destruction of β-cells in the course of insulitis is probably caused by direct contact with activated macrophages and T-cells, and due to the exposure to soluble mediators secreted by these cells, including cytokines, oxygen free radicals and nitric oxide (NO) (2). Indeed, *in insula* overproduction of NO by β-cells themselves is one of the most significant factors that kill β-cells (2), a situation that is very likely to occur in the presence of inflammatory cytokines which activate the transcription of β-cell inducible nitric oxide synthase (NOS2), an enzyme whose expression is nuclear factor κB (NF-κB)-driven and uses L-arginine as substrate (2). On the other hand, macrophages can release, into the inflammatory microenvironment, the enzyme arginase (3), which splits L-arginine into urea and L-ornithine, thus avoiding its conversion into NO and favoring the resolution of the inflammation (4). Interestingly, the depletion of L-arginine itself is sufficient to inhibit T cell proliferation by the downregulation of the ζ-chain, the main signal-transduction component of the T cell receptor (TCR) complex (5). It has also been demonstrated that β-cells possess a cytokine-inducible arginase activity (6), which may account for an expressive reduction in NO synthesis.

If, on the one hand, excessive NO production within β-cells may trigger oxidative/nitrosative stress leading to cell death, NO is, on the other, an obligatory mediator of insulin secretion [see ref. (1) for review]. In fact, it has long been recognized that L-arginine, the immediate precursor of NO, is one of the most powerful insulin secretagogues (7), while L-arginine deficiency is associated with insulinopenia

and failure to secrete insulin in response to glucose (8). NO has been eventually and incontestably shown to be a physiological regulator of insulin secretion in β-cells, in an elegant experimental protocol designed by the Prof. Anne Marie Salapatek's group in Canada, in a seminal paper (9) that deserves deep and meticulous reading.

The presence of an NF-κB-dependent inducible isoform of NO synthase (iNOS, encoded by NOS-2 gene) predisposes β-cells to an enormous susceptibility to develop oxidative/nitrosative stress, because NO synthesis may vary as inflammatory cells and their cytokines are present or not. Since classical antioxidant defenses (*e.g.* superoxide dismutase, catalase) are poorly distributed in β-cells (1), glutathione (GSH) becomes the most important protector of β-cell redox status. However, since the regeneration of GSH from glutathione disulfide (GSSG, its oxidized form) is very weak because β-cells have only a marginal GSSG reductase activity (1), the *de novo* synthesis of GSH from glutamate, cysteine and glycine fulfils the main role in protecting β-cell redox status. This led us to hypothesize that decreased levels of L-arginine for the NO-assisted insulin secretion would determine the shift of intraislet glutamate metabolism from the synthesis of GSH (10) to that of L-arginine, leading to a redox imbalance that activates nuclear factor κB exacerbating inflammation and NO-mediated cytotoxicity (1). In order to assess this possibility, we investigated the *in vitro* effects of the manipulation of L-arginine in the culture media of a clonal insulin-secreting β-cell line (BRIN-BD11) during its exposure to an inflammatory cytokine cocktail which is thought to be similar to that occur at the onset of type 1 diabetes.

RESEARCH DESIGN AND METHODS

Culture of BRIN-BD11 pancreatic β-cells, pro-inflammatory cocktail challenge and measurement of insulin secretion at different L-arginine concentrations. The

rat clonal insulin-secreting β-cell line BRIN-BD11 was chosen because its metabolic, signaling, insulin secretory and cell viability responses to glucose, amino acids as well as other stimuli have already been well characterized (10-12). BRIN-BD11 cells were maintained in T175 sterile tissue culture flasks in 50-70 mL of RPMI-1640 culture medium supplemented with 10% (v/v) FCS, 0.1% (v/v) antibiotics (100 U/mL penicillin and 0.1 mg/mL streptomycin) and 11.1 mmol/L D-glucose, pH 7.4, at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells were subsequently seeded into six-well plates (0.75x10^5 cells per well), allowed to adhere overnight and then washed with PBS after which they were incubated in fresh media, containing 11.1 mM D-glucose, 2 mM L-glutamine, in the presence or absence of a sub-lethal pro-inflammatory cytokine cocktail (IL-1β 0.3125 U/mL, TNFα 31.25 U/mL, IFNγ 15.625 U/mL) (13) at different concentrations of L-arginine (1.15 mM and 5 mM). This cocktail has been shown to mimic the onset of insulitis of type 1 diabetes (13). After 24 h, an aliquot of the medium was removed, centrifuged at 200 g for 5 min at 4°C and used for quantization of insulin or, at 16,000 g for 10 min at 4°C for the determination of metabolites. In another set of experiments, cells were cultured for 24 h under the above conditions and then maintained for 40 min in the presence of 1.1 mmol/L glucose to be acutely-stimulated for 20 min in the presence of 16.7 mM glucose and 10 mM alanine (a stimulus that results in a robust and reproducible secretory response at normal conditions (14)). Afterwards, aliquots from incubation medium were taken and centrifuged at 200 g for 5 min at 4°C to be analyzed for acute insulin secretion using the Mercodia UltrasenstiveRat Insulin ELISA kit.

Cell viability measurements. Cell viability was determined using the cell proliferation reagent WST-1, a tetrazolium salt that is cleaved by mitochondrial dehydrogenases in

viable cells. Briefly, 100μL of cell suspension (containing 2×10^4 cells) were plated in each well of 96-well plates. After a 24-hour reattachment period in culture, the cells were treated with a sub-lethal dose (above) of pro-inflammatory cytokine cocktail for further 24h. At the end of each experiment, the cell proliferation reagent WST-1 (10μL) was added to each well and the cells were incubated at 37°C for 1.5 h to be spectrophotometrically analyzed at 450 nm. Cell viability was *ca.* 97% with no observable changes with respect to any treatment described in this study after 24 h of incubation and further periods of metabolite stimulation.

Enzymatic determination of metabolites. *Urea production-* Urea was measured in 50-μL samples using the 96-well QuantiChromTM Urea Assay Kit (DIUR- 500) according to manufacturer's instructions. *Nitrite Production-* The production of NO by BRIN-BD11 cells was assessed by measuring, in the culture medium, the formation of nitrites which were determined by using the Griess Reagent System (Promega Medical Supply Co.). *Glucose and glutamine consumption and production of glutamate -* Glucose, glutamine and glutamate concentrations were measured in the incubation medium using an YSI 7100 amino acid analyzer (YSI Incorporated). Briefly, an enzyme specific for the substrate of interest is immobilized between two membrane layers, polycarbonate and cellulose acetate. The substrate is oxidized as it enters the enzyme layer, producing hydrogen peroxide, which passes through cellulose acetate thus reaching a platinum electrode where the hydrogen peroxide is oxidized. The resulting current is proportional to the concentration of the substrate.

Measurement of glutathione (GSH) and glutathione disulfide (GSSG) content. BRIN-BD11 cells were seeded into six-well plates (2×10^6 cells/well), allowed to

adhere overnight and then washed with PBS after which they were incubated in fresh media, containing 11.1 mM D-glucose and 2 mM L-glutamine, in the presence or absence of sub-lethal pro-inflammatory cytokine cocktail (above). Afterwards, cells were rinsed twice with PBS and disrupted in 200 µL of 5% (w/v) metaphosphoric acid on ice. After centrifugation (16,000 x g, 5 min at room temperature), cell lysates were spectrophotometrically (415 nm) assayed on a temperature-controlled microplate reader (Molecular Devices SpectraMax Plus 384) by a modification of the 5,5'-dithiobis(2-nitrobenzoic acid) [DTNB]/GSSG reductase recycling method, using the N-ethylmaleimide conjugating technique for GSSG sample preparation (15). Samples (10 µL) were assayed in 105-µL final volume in 96-well polystyrene plates at 37°C in the presence of 10 mM DTNB, 0.17 mM β-NADPH (dissolved in 0.5% (w/v) NaHCO$_3$ as a stabilizing agent) and 0.5 U/mL GSSG reductase (EC 1.6.4.2).

Preparation of protein extracts from BRIN-BD11 cells. BRIN-BD11 cells were lysed in 150 µL of RIPA lysis buffer (MSC, Dublin, Ireland) containing protease inhibitors. Cell lysates were transferred to fresh ice-cold microcentrifuge tubes and were then placed on a shaker at 4°C for 15 min. The cells were then centrifuged at 14,000 g for 15 min at 4°C. The supernatant fraction was placed in a fresh tube and stored at −20°C. Cellular protein was determined by using a BCA protein assay kit (Pierce, Rockford, IL, USA kit no. 23225), which utilizes a modification of the biuret reaction.

Western blot analysis. Cells were seeded into 6-well plates (1.5 x 10^6 cells/well), allowed to adhere overnight and then washed with PBS after which they were incubated in fresh media, containing 11.1 mM D-glucose, 2 mM L-glutamine, in the presence or absence of the pro-inflammatory cytokine cocktail (above) for further 24-h. After

culture and cell lysis, equal amounts of BRIN-BD11 cell protein extracts were prepared and subsequently subjected to 10% SDS-PAGE and electrophoretically transferred onto a nitrocellulose sheets. The sheets were blocked in 5% milk protein and probed with polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) anti-NOS2, AMP kinase (AMPK), phosphorylated AMPK (AMPK-P) and heme-oxygenases 1 and 3 (HO-1, HO-3, respectively). The blots were washed and visualized with a horseradish peroxidase-based Supersignal West Pico chemiluminescent substrate (Pierce). Results of digitalized images were expressed as the means ± S.D. using GAPDH as an expression control.

Statistical analysis. Paired and unpaired bitailed Student's *t*-test were used as appropriate to evaluate the statistical significance of differences between two group means, and ANOVA was used for multiple comparisons. Data are presented as means ± S.D. and differences were considered significant at a P value of <0.05.

RESULTS

Effect of increased L-arginine concentrations on glutathione metabolism, glutamate production and glutamine consumption in BRIN-BD11 cells challenged with pro-inflammatory cytokines. Increasing the concentrations of L-arginine from 1.15 to 5.0 mM in the culture medium induced a huge augment in GSH contents of BRIN-BD11 cells (**Fig. 1A**; from 0.279 ± 0.037 to 0.596 ± 0.044 μmol/mg protein/24h). Even in the presence of the cytokine cocktail, which *per se* reduced BRIN-BD11 intracellular GSH by 44% in 1.15-mM L-arginine treated cells, changing to 5 mM L-arginine was able to increase GSH contents by more than 87%. In line with the above results, enhancing L-arginine from 1.15 to 5 mM produced a remarkable reduction in

GSSG contents (from 0.168 ± 0.009 to 0.021 ± 0.007 μmol/mg protein/24h) and was also capable to partially revert the marked rise in GSSG levels evoked by sub-lethal concentrations of the pro-inflammatory cocktail, causing GSSG contents almost to return to control reference levels (**Fig. 1B**). As a consequence, enhancing L-arginine in the culture media dramatically improved BRIN-BD11β-cell redox status, as GSSG to GSH ratio was expressively reduced in both control (from 0.58 ± 0.058 to 0.036 ± 0.011 μmol/mg protein/24h) and cytokine-challenged (from 2.930 ± 0.027 to 1.005 ± 0.283 μmol/mg protein/24h) groups (**Fig. 1C**).

Figure 1. Effect of increased l-arginine concentrations on glutathione metabolism, glutamate production and glutamine consumption in BRIN-BD11 cells challenged with pro-inflammatory cytokines for 24 h. Clonal insulin-secreting BRIN-BD11 cells were seeded into 6-well plates (2 x 106cells/well) and allowed to adhere overnight and then washed with PBS after which they were incubated in fresh media, containing 11.1 mM d-glucose, 2 mM l-glutamine, in the absence or presence of a sub-lethal pro-inflammatory cytokine cocktail. After 24 h, cells were rinsed twice with PBS and disrupted in 5% (w/v) metaphosphoric acid (MPA) on ice to be spectrophotometrically assayed for GSH (A) and GSSG (B). The index of redox status (GSSG to GSH ratio) was also assessed (C). Glutamate production (D) and glutamine consumption (E) was evaluated in β-cell culture media after 24 h in culture by using an YSI 7100 amino acid analyzer. White bars: 1.15 mM l-arginine; black bars: 5 mM l-arginine. Data are means ± sd of at least 5 separate preparations in double. For the comparisons: *P<0.05, 1.15 mM *vs* 5.0 mM group; †P<0.05, control *vs* cytokine-treated group at the same l-arginine concentration; ‡P<0.05, control *vs* cytokine-treated group at the different l-arginine concentrations.

Since glutamate is an immediate precursor of GSH (γ-glutamylcysteinyl-glycine) and glutamine can be utilized by β-cells to generate both glutamine and L-arginine

(1),we investigated how glutamate production and glutamine consumption could be influenced by L-arginine manipulation. Paralleling the increments in GSH, changing L-arginine concentrations induced a rise in the production of glutamate by BRIN-BD11 cells in both control (47%) and cytokine-treated (78%) groups (**Fig. 1D**). Interestingly, cytokine-stimulation significantly enhanced glutamate production only cells cultured in the presence of 5.0 mM L-arginine but not in lower concentration. Conversely, changing L-arginine concentrations from 1.15 to 5.0 mM significantly reduced glutamine consumption by about 20% in control cells but did not affect cytokine-treated cells (**Fig. 1E**). Curiously, although statistically significant, the effects of the cytokine cocktail on BRIN-BD11 cells were minimal.

Figure 2. Effect of increased l-arginine concentrations on insulin secretion, glucose consumption and lactate production by BRIN-BD11 cells challenged with pro-inflammatory cytokines for 24 h. Cells were seeded into 24-well plates (0.75 x 105 cells/ well), allowed to adhere overnight and then washed with PBS after which they were incubated in fresh media, containing 11.1 mM d-glucose, 2 mM l-glutamine, in the absence or presence of a sub-lethal pro-inflammatory cytokine cocktail. After 24 h, aliquots of the media were removed and used for quantitation of insulin by ELISA (A). Glucose consumption (B) and lactate production (C) were also measured by using an YSI 7100 amino acid analyzer. White bars: 1.15 mM l-arginine; black bars: 5 mM l-arginine. Data are means ± sd. For the comparisons: *P<0.05, 1.15 mM *vs* 5.0 mM group; †P<0.05, control *vs* cytokine-treated group at the same l-arginine concentration; ‡P<0.05, control *vs* cytokine-treated group at the different l-arginine

Effect of increased L-arginine concentrations on insulin secretion, glucose consumption and lactate production by BRIN-BD11 cells challenged with pro-inflammatory cytokines. As depicted in **Fig. 2A**, L-arginine manipulation did not influenced insulin secretion in BRIN-BD11 β-cells cultivated for 24 h. Also, the well-known and expected suppression of insulin secretion in cytokine-treated cells was not reversed by L-arginine. However, L-arginine conspicuously increased glucose consumption in the same period (*ca.*80-fold) being this rise even higher in cytokine-treated cells (**Fig. 2B**). Interestingly, this sort of L-arginine-dependent uncoupling of the stimulus-secretion link was not accompanied by an elevation of lactate production. On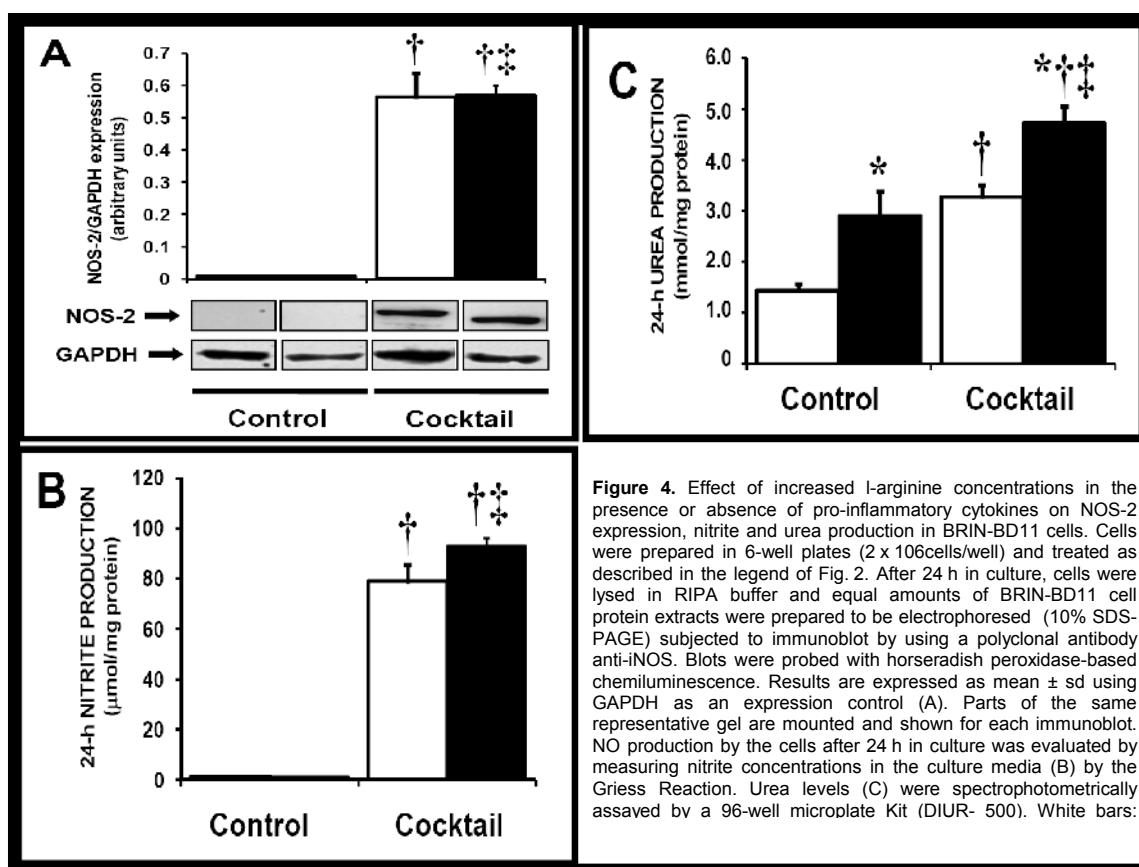 the contrary, as shown in **Fig. 2C**, L-arginine indeed decreased lactate formation in both control and cytokine-treated cells by up to 22%. The response of BRIN-BD11 β-cells to a powerful nutrient stimulus (16.7 mM glucose plus 10 mM alanine for 20min) following the first 24-h period in culture was depressed in cytokine-treated cells at both L-arginine concentrations employed (**Fig. 3**), similarly to previous results from our group (13). Indeed, during this further 20-min incubation period, regardless a previous exposure or not to the pro-inflammatory cytokines, basal insulin secretion was maintained in all experimental groups, except in 5.0 mM L-arginine group, where a 63%-reduction of secretion was observed. This suggests that L-arginine may be involved in a mechanism of metabolic desensitization, which led us to investigate three key metabolic points that could be related with the above findings: 1) that L-arginine could interfere in NO-assisted insulin secretion via modulation of iNOS or arginase pathway; 2) that L-arginine could favor some NO-dependent metabolic pathway critical for the maintenance of intracellular glucose homeostasis, such as AMPK, since recent studies from this laboratory indicated that NO induces the activation of AMPK phosphorylation (Krause et al., unpublished work); 3) that L-arginine could divert

glucose metabolism towards intracellular pathways that are able to save GSH and improve cellular antioxidant defense. These possibilities were checked and the results are shown below.

Figure 3. Effect of 24‑h culture with increased l-arginine concentrations in the presence or absence of pro-inflammatory cytokines on acutely metabolite-stimulated insulin secretion by BRIN-BD11 cells. Cells were prepared and treated as described in the legend of Fig. 2. After 24 h, cells were washed twice with PBS and then, acutely incubated for 40 min in the presence of 1.1 mmol/L glucose followed by an additional 20‑min period in the presence of 16.7 mM glucose and 10 mM alanine when aliquots of the incubation medium were removed and analyzed for insulin by ELISA. White bars: 1.15 mM l‑arginine; black bars: 5 mM l‑arginine. Data are means ± sd of at least 5 separate preparations in double. For the comparisons: ∗P<0.05, 1.15 mM *vs* 5.0 mM group; †P<0.05, control *vs* cytokine-treated group at the same l‑arginine concentration; ‡P<0.05, control *vs* cytokine-treated group at the different l‑arginine concentrations.

Effect of increased L-arginine concentrations on NOS-2 expression and production of NO and urea. After 24 h in culture, cytokine treatment evoked a dramatic increase in NOS-2 expression (**Fig. 4A**) which was paralleled by a similar rise in NO production, as inferred from nitrites delivered in the culture medium: from undetectable levels to 78.98 ± 6.27 and 93.03 ± 2.86 μmol/mg protein/24h, at 1.15 and 5.0 mM L-arginine concentrations, respectively (**Fig. 4B**). While this was an expected result, since the pro-inflammatory cytokines tested are well known to induce NF-κB activation, and, consequently, NOS-2 protein expression, it jumps out that enhancing L-arginine concentration from 1.15 to 5.0 mM in the medium had absolutely no effect on NO production, thus suggesting that the conversion of L-arginine into NO and L-citrulline should be saturated in respect to NOS-2. Conversely, increased L-arginine concentration

markedly enhanced urea production (**Fig. 4C**), in both control (103%) and cytokine-treated (45%) cells, convincingly indicating that excess L-arginine was drifted towards L-ornithine/glutamate pathway through th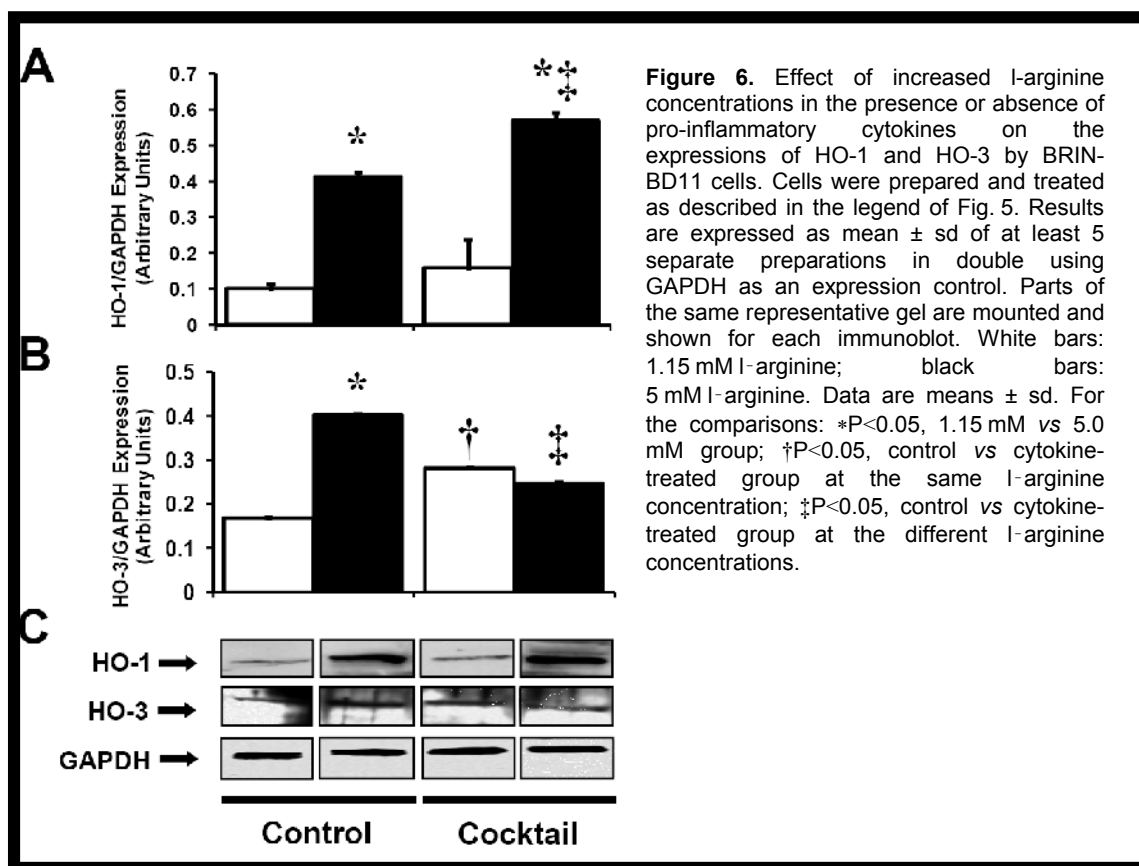e concerted action of pyrroline-5-carboxylate dehydrogenase (PCDH) and ornithine-carbamoyltransferase (OCT) and explains the rise in glutamate production (**Fig. 1D**) in the absence of glutamine consumption (**Fig. E**) under the same conditions.

Figure 4. Effect of increased l-arginine concentrations in the presence or absence of pro-inflammatory cytokines on NOS-2 expression, nitrite and urea production in BRIN-BD11 cells. Cells were prepared in 6-well plates (2 x 106cells/well) and treated as described in the legend of Fig. 2. After 24 h in culture, cells were lysed in RIPA buffer and equal amounts of BRIN-BD11 cell protein extracts were prepared to be electrophoresed (10% SDS-PAGE) subjected to immunoblot by using a polyclonal antibody anti-iNOS. Blots were probed with horseradish peroxidase-based chemiluminescence. Results are expressed as mean ± sd using GAPDH as an expression control (A). Parts of the same representative gel are mounted and shown for each immunoblot. NO production by the cells after 24 h in culture was evaluated by measuring nitrite concentrations in the culture media (B) by the Griess Reaction. Urea levels (C) were spectrophotometrically assayed by a 96-well microplate Kit (DIUR- 500). White bars:

Effect of increased L-arginine concentrations on the expression of AMP kinase and heme-oxygenases. As shown in **Fig. 5**, enhancing L-arginine concentrations from 1.15 to 5.0 mM increased the appearance of the phosphorylated form of AMPK (the active form, AMPK-P) in both control (89%) and cytokine-stimulated (75%) β-cells cultured for 24 h (**Fig. 5A**), promoting a reduction in the amount of the inactive

unphosphorylated form to similar extent (**Fig. 5B**). Moreover, the increasing effect of

L-arginine over immunodetected AMPK-P protein was even more accentuated (38%

higher) in cytokine-challenged cells as compared to controls. During the same 24-h

period of cultivation, the expressions of the redox-protecting enzymes HO-1 (inducible

form of HO) and HO-3 (the constitutive form) were also evaluated. The increment in L-

arginine from 1.15 to 5 mM produced a remarkable enhancement in the expression of

HO-1, in both control (4.1-fold) and cytokine-treated (3.6-fold) β-cells (**Fig. 6A**). It is

of note that, in the presence of the pro-inflammatory cytokine cocktail, the increase in

HO-1 expression caused by changing L-arginine from 1.15 to 5 mM was found to be

even higher than that observed in control cells (an elevation of 38%). On the other hand,

HO-3 expression was affected by L-arginine increment only in control β-cells, where it

was found a 2.4-fold rise of expression (**Fig. 6B**).

Figure 5. Effect of increased l-arginine concentrations in the presence or absence of pro-inflammatory cytokines on the expressions of AMPK and its phosphorylated form (AMPK-P) by BRIN-BD11 cells. Cells were seeded into 6-well plates (1.5x106 cells per well) and prepared as described in the legend of Fig. 2. After 24 h in culture, cells were lysed in RIPA buffer and equal amounts of BRIN-BD11 cell protein extracts were prepared to be electrophoresed (10% SDS-PAGE) subjected to immunoblot by using a polyclonal antibody anti-AMPK and AMPK-P. Blots were probed with horseradish peroxidase-based chemiluminescence. Results are expressed as mean ± sd of at least 5 separate preparations in double using GAPDH as an expression control. Parts of the same representative gel are mounted and shown for each immunoblot. White bars: 1.15 mM l-arginine; black bars: 5 mM l-arginine. Data are means ± sd. For the comparisons: ∗P<0.05, 1.15 mM *vs* 5.0 mM group; †P<0.05, control *vs* cytokine-treated group at the same l-arginine concentration; ‡P<0.05, control *vs* cytokine-treated group at the different l-arginine concentrations.

Figure 6. Effect of increased l-arginine concentrations in the presence or absence of pro-inflammatory cytokines on the expressions of HO-1 and HO-3 by BRIN-BD11 cells. Cells were prepared and treated as described in the legend of Fig. 5. Results are expressed as mean ± sd of at least 5 separate preparations in double using GAPDH as an expression control. Parts of the same representative gel are mounted and shown for each immunoblot. White bars: 1.15 mM l-arginine; black bars: 5 mM l-arginine. Data are means ± sd. For the comparisons: ∗P<0.05, 1.15 mM vs 5.0 mM group; †P<0.05, control vs cytokine-treated group at the same l-arginine concentration; ‡P<0.05, control vs cytokine-treated group at the different l-arginine concentrations.

DISCUSSION

The intrinsic nature of β-cell metabolism that generates NO via an NF-κB-dependent enzyme (iNOS), which is inducible under inflammatory conditions, unremittingly predisposes β-cells to oxidative stress. Alongside of this, it is of note that β-cells present a very low level of expression of antioxidant enzymes such, as catalase and GSH peroxidase (GSPx), compared with other tissues. Hence, β-cells are prone to oxidative/nitrosative stress, particularly because this cell type generates NO and shows a very accelerated mitochondrial flux of electrons (and consequently, elevated tendency towards the production of reactive oxygen and nitrogen species, ROS/RNS) during glucose-stimulated insulin release (9; 16). This is the case in the pre-diabetic condition where the antioxidant status appears to be impaired (17) and, due to such a vulnerability

in antioxidant activity, it is often observed significant increases in lipid hydroperoxides, conjugated dienes and protein carbonyls, which are markers for oxidative stress (18). Hence, the low antioxidant defence in certain individuals (even if transiently) may make them susceptible to an enhanced oxidative stress and the eventual β-cell death that categorize the onset of type 1 diabetes. Therefore, GSH becomes the main resource of antioxidant defense for β-cells. GSH is a strong reductant *per se* and an antioxidant agent that does not exclusively depend on enzyme-catalyzed reactions to maintain a reducing environment.

In order to adequately provide GSH for antioxidant defense, β-cells may either regenerate GSH from GSSG via a GSSG reductase (GSRd)-catalyzed reaction or synthesize it, via *de novo*, through the concerted action of γ-glutamylcysteine synthetase (γ-GCS, also known as glutamate-cysteine ligase) and GSH synthetase, which are ATP-consuming enzymes. Regeneration of GSH from GSSG, which utilizes NADPH as a co-factor but does not require ATP, is metabolically less expensive than the *de novo* synthesis from the constituent amino acids. However, differently from the majority of cell types, pentose phosphate shunt activity is relatively low in β-cells (19), which is exacerbated by the high flux of glucose towards ATP production that empty glucose-6-phosphate dehydrogenase (1). Therefore, NADPH must be obtained from the cytosolic malic enzyme ($NADP^+$–dependent malate dehydrogenase), capable of converting malate to pyruvate with the concomitant production of NADPH from $NADP^+$ (20). The *de novo* GSH synthesis, on the other hand, is completely dependent on the supply of glutamate, not only because this amino acid itself takes part of GSH molecule, but also because glutamate generates serine that produces cysteine after reacting with methionine, and glycine, via a reaction with glycolysis-derived 3-phosphoglycerate. However, muscle-derived glutamine and alanine are the major sources of glutamate for

the β-cells, since the liver releases glutamate into the blood at concentrations that are relatively much lower respective to those amino acids. In fact, previous reports from this laboratory have highlighted the importance of alanine and glutamine for GSH generation, insulin secretion and protection against pro-inflammatory cytokines (10; 14; 21).

The observation that L-arginine is able to increase GSH synthesis (**Fig. 1**), regardless the presence or absence of pro-inflammatory cytokines, sheds light on an as yet unpredicted facet of β-cell metabolism: that L-arginine is a direct precursor of GSH. This is reinforced by the finding that L-arginine enhances glutamate production (**Fig. 1D**) without rising the consumption of glutamine (**Fig. 1E**). This scenario is illustrated in **Fig. 7A** in which, under normal L-arginine concentrations, glutamine and alanine are the principal resources of glutamate for GSH synthesis, while L-arginine is used to replenish iNOS for NO synthesis necessary for insulin secretion. Enhancing L-arginine availability allows for the increment of the metabolic flux of the left side of β-cell urea cycle which furnishes glutamate by coupling the consumption of L-ornithine via PCDH and OCT. Even when β-cells are challenged with pro-inflammatory cytokines, a situation that dramatically enhances the expression of iNOS and the flux of L-arginine towards the production of huge amounts of NO, the treatment with enhanced L-arginine concentration was still capable of providing redox protection, as estimated by GSSG to GSH ratio (**Fig. 1C**). Intracellular redox potential, *i.e.* the redox "voltage", given by the Nernst equation, may be written as $\varepsilon = \varepsilon_o - (RT/nF).\ln([GSSG]/[GSH])$ under physiological conditions. Because of this, [GSSG]/[GSH] ratio is considered the index of cellular redox status (22), and gives a panorama of how intracellular redox reactions may be affected if one or both of the components of this quotient have changed.
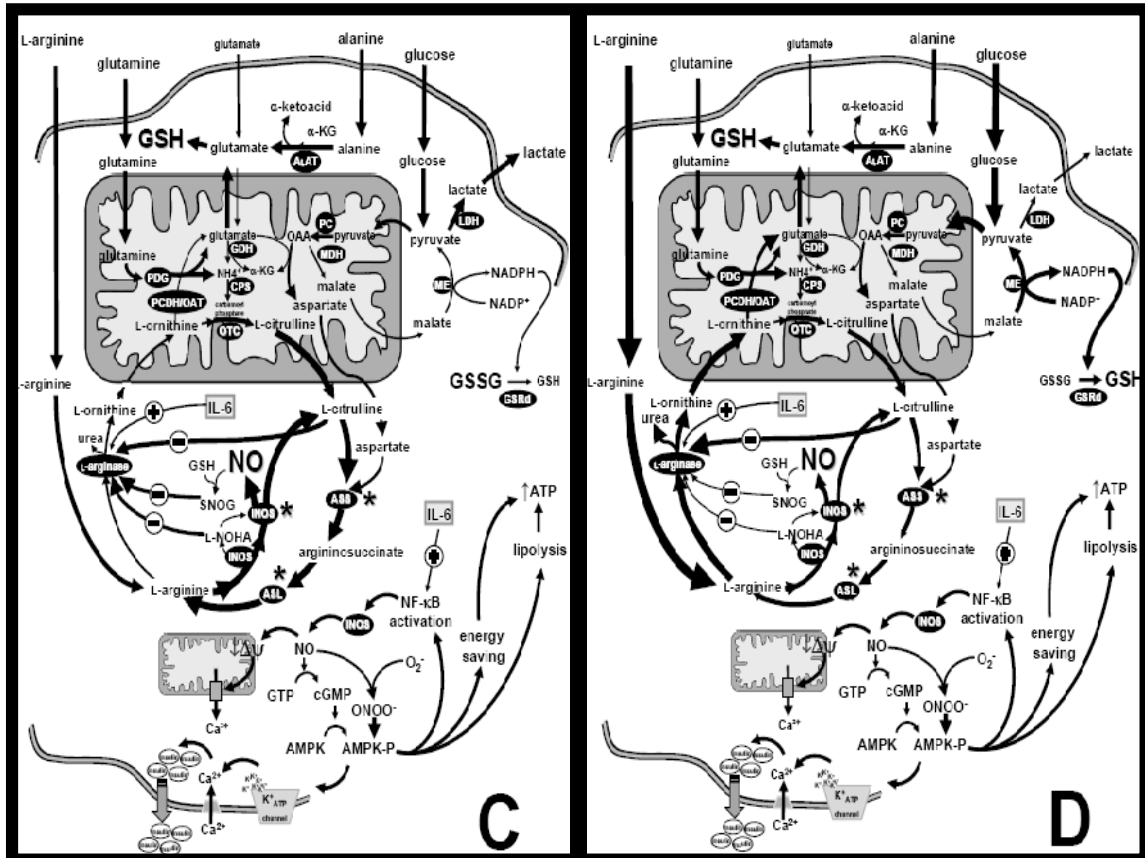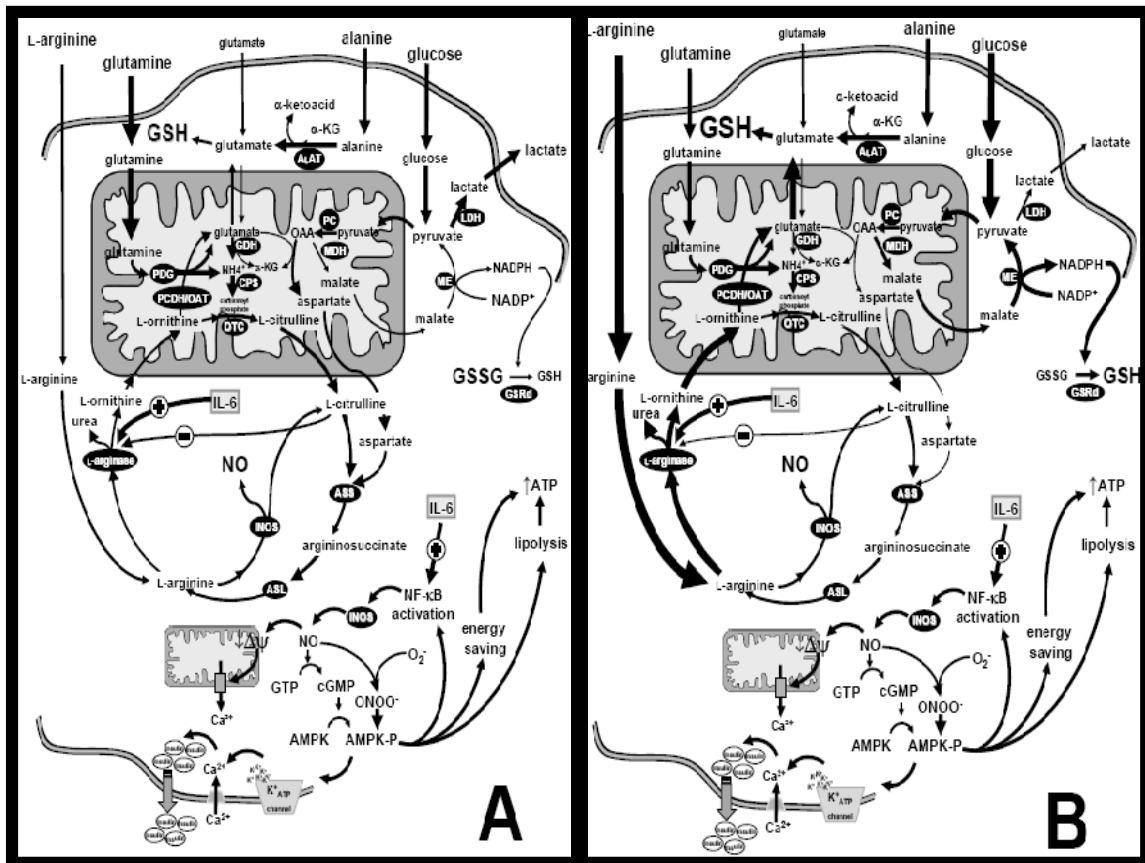
Figure 7. L-Arginine-glutamate-NO coupling in β-cells. Under physiological secretagogue-mediated insulin release, both NO and GSH are obligatory intermediates. Accordingly, β-cells have an intricate iNOS-centred machinery to produce NO, which potentiates insulin secretion physiologically. At the same time, insulin-secreting pancreatic cells utilize glutamate-derived GSH in order to maintain redox status needed to allow hormonal secretion and to avoid a possible NO-mediated cytotoxicity. L-Arginine derived from the kidney is the physiological substrate for the NF-κB-dependent iNOS-catalyzed NO production in β-cells. Under insufficient L-arginine supply, however, the high throughput of NO for β-cells may be attained by the concerted action of phosphate-dependent glutaminase (PDG), glutamate dehydrogenase (GDH), aspartate aminotransferase (not shown), carbamoylphosphate synthetase (CPS), ornithine transcarbamoylase (OTC), argininosuccinate synthetase (ASS) and argininosuccinate lyase (ASL), which, dramatically enhances the flux of glutamate towards NO production. In the presence of an inflammatory NF-κB-centered cytokine insult, multiple negative feedback systems act in β-cells in order to warrant L-arginine entry in iNOS metabolic pathway (lower part of the figure). This is achieved mainly due to the inhibition of L-arginase activity by L-citrulline, N^G-hydroxy- L-arginine (L-NOHA, an intermediate in NO synthesis) and S-nitrosoglutathione (SNOG), which is formed during NO biosynthesis. On the other hand, β-cells have to synthesize GSH from glutamate, cysteine and glycine, because regeneration of GSH from glutathione disulfide (GSSG) via NADPH-dependent GSSG reductase is relatively low in β-cells because of the high flux of glucose towards ATP production that empty pentose-phosphate shunt impairing NADPH production. In turn, *de novo* GSH synthesis is mainly dependent on liver-emanated supply of glutamate, which is not enough to allow for the enormous flux towards γ-glutamylcysteine synthetase and GSH synthetase in the GSH biosynthetic pathway. Therefore, muscle-derived alanine and glutamine constitute the principal sources of glutamate for GSH synthesis. Because of this, any reduction in L-arginine supply to β-cells accounts for a rapid shift in glutamate metabolism from GSH synthesis towards NO production. For instance, during Th1-elicited immune responses, the concerted enhancement of NF-κB-mediated (*) expression of ASS, ASL and iNOS dramatically boosts NO production from glutamate. If this rise in NO production is not accompanied by an enhanced L-arginine supply to β-cells, NO becomes very cytotoxic. Type 2 cytokines, such as interleukim-6 (IL-6) may alleviate NO toxicity by enhancing L-arginase expression that deviates L-arginine to the formation of L-ornithine and urea. Results from the present work reveal a novel as yet unpredicted facet of L-arginine metabolism in that an increase in its plasma concentrations (from A to B) could drift GSH metabolism from its original main source, via glutamine metabolism, towards the production of glutamate via the left side of the β-cell urea cycle, by the consecutive action of L-arginase, pyrroline-5-carboxylate dehydrogenase (PCDH), ornithine aminotransferase (OAT), γ-glutamylcysteine synthetase (not shown) and GSH synthetase (not shown). Under inflammatory stimuli (C and D), enhancement of L-arginine concentration may alleviate the excessive flux through iNOS by limiting L-arginine availability due to its conversion into GSH. Concomitantly, elevation of L-arginine levels are thought to deviate glucose mitochondrial metabolism towards its cytosolic utilization as a NADPH precursor via malic enzyme (ME). This favors the regeneration of more GSH molecules from GSSG under oxidative stress conditions. L-Arginine may also stimulate AMPK activation which modulates closure of K_{ATP} channels and insulin secretion. NO is also capable of activating AMPK. However, in a high L-arginine environment, the excessive activation of AMPK may stimulate lipolysis and energy saving at the expense of insulin secretion. Arrow widths indicate the intensity of the metabolic flux through each pathway.

The finding that L-arginine conspicuously enlarge glucose consumption in β-cells, which was enhanced in the presence of a pro-inflammatory cytokine insult

(**Fig. 2B**), is absolutely novel and may suggest that L-arginine is able to deviate glucose from mitochondrial metabolism towards the formation of NADPH via cytosolic malic enzyme, since pentose phosphate shunt has poor activity in β-cells. This proposition is supported by the fact the lactate production was reduced by the augmentation in L-arginine in both control and cytokine groups (**Fig. 2C**). Indeed, we believe that, under high argininemia, glutamate is only marginally conducted to L-arginine via the right side of the urea cycle to generate NO in β-cells, unburdening GSH synthesis from glutamate which liberates oxalacetate to be converted into malate (**Fig. 7B and C**). At the same time, L-arginine remarkably enchanced the conversion of AMPK into its active phosphorylated form (**Fig. 5**), thus favoring fatty acid oxidation when glucose is supporting NADPH for GSH regeneration. This occurred, however, at the expense of stimulus-secretion coupling since, although L-arginine did not affect chronic insulin secretion during 24 h in culture (**Fig. 2A**), metabolite-stimulated insulin secretion was inhibited, particularly in cytokine treated β-cells (**Fig. 3**).

We have previously reported that the exposure of pancreatic β-cells to a sub-lethal concentration of pro-inflammatory cytokines (IFN-γ, TNF-α, IL-1β) is able to shift β-cell metabolism away from a key role in stimulus–secretion coupling and towards a catabolic state which may have been related to cell defense (13). A machinery to prevent massive NO production, such as high arginase activity (6) in a very active urea cycle, allows for β-cells to have NO-dependent secreting function even in the presence of very low antioxidant defenses, as we have previously hypothesized (1).

Interestingly, previous studies with skeletal and cardiac muscle, brain, liver and adipose tissue have shown that L-arginine supplementation, in male Zucker diabetic fatty rats, powerfully induces the activation of HO-3, AMPK, and PGC-1α (PPARγ co-

activator 1α, which exert a high potential to boost mitochondrial biogenesis and increase oxidative metabolism) (23). It has also been suggested that changes in AMPK activity may contribute to the regulation of insulin secretion (24), while previous studies from this laboratory have shown that palmitate activates AMPK and acutely potentiates insulin secretion from isolated rat islets and MIN6 beta cells X. Moreover, a recent study has shown that the AMPK agonist AICAR dose-dependently improves β-cell function reducing apoptosis induced by prolonged hyperglycemia X. Therefore, the activation of AMPK by L-arginine may induce, not only an increment in oxidative metabolism which drives ATP production, but may also prevent β-cell glucolipotoxicity.

When NOS-2 expression was stimulated by the cytokine cocktail, NO synthesis was not enhanced by changing L-arginine in the culture medium (**Fig. 4A and B**), a result that suggests that iNOS should be saturated regarding L-arginine which, in turn, was converted to urea (**Fig. 4C**). This shunt in L-arginine metabolism efficiently preserves β-cell redox status by favoring the production of more GSH molecules under situations of excessive NO production.

Arginases usually present K_m values that are much higher than those found for iNOS but V_{max} of arginases are also much higher than that of iNOS (25), so that V_{max}/K_m ratios of both types of enzymes are close to each other and these enzymes are expected to compete for L-arginine nearly to equilibrium in the cells. In our hands, iNOS seemed to be saturated in β-cells regarding the presence of inflammatory cytokines or not, so that β-cell urea cycle is able to furnish glutamate for the GSH synthesis. Moreover, L-arginine may protect β-cells in another front that is the induction of HO-1 expression. HO activity is an important detoxifying enzyme due to its ability to

scavenge heme groups thus providing redox protection (26). However, it is plausible that HO expression in β-cells in response to L-arginine may also play a metabolic role, since one of its direct products, carbon monoxide (CO), has been recently pointed out to induce insulin secretion and to improve *in vivo* function of β-cells after transplant (26). Moreover, the long-lasting expression of this enzyme has been shown to delay the progression of type 1 diabetes in NOD mice X. Hence, L-arginine can be recognized as an antioxidant in its own right, being comparable with known antioxidant stimuli, such as phytochemical supplements (27).

ACKNOWLEDGEMENTS

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5. CONCLUSÕES E RECOMENDAÇÕES

A diabetes tipo I apresenta incidência de apenas 5 a 10% de todos os casos de diabetes, no entanto, este número continua a aumentar pelo mundo inteiro e apresenta sérias implicações a curto e em longo prazo. Além disso, os pacientes acometidos da patologia precisam ser tratados cronicamente com insulina desde a infância até o restante de suas vidas. Não bastassem esses inconvenientes, a administração crônica do hormônio leva, muitas vezes, a respostas imunológicas contra a insulina xenobiótica (porcina, bovina) enquanto que o custo de insulinas "humanizadas" ou recombinantes é muito elevado. Por estas razões, muitos pacientes, especialmente das classes sociais mais baixas, acabam tendo sérios comprometimentos cardiovasculares, neurológicos e metabólicos que, muitas vezes, culminam com o coma hiperglicêmico e morte prematura.

O tratamento de pacientes diabetes tipo I com insulina é a única opção eficaz atual, uma vez que as numerosas tentativas com agentes supressores ou moduladores da função imune têm falhado na tentativa de preservação das células β em longo prazo. Atualmente, entre as terapias concorrentes no tratamento do diabetes, o exercício físico regular representa uma alternativa de baixo custo e eficaz no que concerne a melhora metabólica e imunológica do indivíduo portador de diabetes, tanto do tipo I quanto do tipo II.

Apesar de o exercício físico apresentar uma série de efeitos benéficos para a homeostase glicêmica, como redução da resistência periférica à insulina e melhor aproveitamento do metabolismo hepático de carboidratos, a possibilidade de que o exercício possa prevenir a instalação do diabetes tipo I através da ação do controle de liberação de citocinas anti e pró-inflamatórias bem como através da mobilização de importantes aminoácidos para a manutenção normal da função de células beta, como a arginina e a glutamina, nunca havia sido abordada.

Conforme sugerido por nosso grupo, a origem auto-imunológica do diabetes do tipo I pode ser iniciada através da ativação do sistema simpático-CRH-histamina que culmina em ativação de linfócitos Th1, grandes consumidores de glutamina, resultando em liberação de citocinas pró-inflamatórias e concomitante redução dos níveis deste aminoácido. O que segue a este processo é uma redução de oferta de L-citrulina para a síntese de L-arginina pelo rim, ocasionando uma redução de L-arginina para a

circulação. Conforme discutido anteriormente, enquanto a glutamina é fonte de precursores de glutationa para a célula beta, a L-arginina é necessária para a síntese, em níveis fisiológicos, de óxido nítrico, para a secreção normal de insulina. Consequentemente, na ausência de L-arginina, o metabolismo da glutamina na célula beta é desviado da síntese do principal antioxidante, a GSH, para a síntese *de novo* de L-arginina e NO. O resultado é uma diminuição das defesas antioxidantes, modificação do estado redox celular que culmina na ativação do NF-κB, exacerbando a citotoxicidade e levando a morte celular.

Embora a hipótese de que o eixo simpático-CRH-histamina ainda não tenha sido demonstrada, outra via fisiológica para a redução da oferta de L-arginina para a célula beta foi sugerida por nosso grupo: a secreção ativa da enzima arginase por macrófagos ativados no microambiente da ilhota.

O envolvimento dos macrófagos como células-chave desencadeadoras das doenças auto-imunológicas já foram demonstrados. Embora a secreção de arginase pro macrófagos tenha sido apontada como um mecanismo em favor da inflamação, por reduzir a competição entre a NOS2 e a arginase pela L-arginina, várias evidencias apontam que sua função é justamente oposta. A secreção de arginase resulta em depleção de L-arginina extracelular (principal fonte para a produção de óxido nítrico pela NOS2) o que levaria a uma redução na produção deste gás. Além disso, a deficiência de arginina leva a uma redução na atividade e proliferação de linfócitos, conforme já discutido no trabalho anterior. Por fim, apesar desta secreção de arginase buscar a resolução da inflamação ela tem, como conseqüência fatídica, uma redução da disponibilidade de arginina para as células beta, o que resultaria na mudança metabólica sugerida em nossa hipótese: redução de GSH e aumento da citotoxicidade, levando a insulite, morte celular e diabetes tipo I.

No presente trabalho, onde modificamos a disponibilidade de L-arginina para células beta expostas a uma condição similar ao inicio do diabetes tipo I (no que concerne a presença de citocinas pró-inflamatórias), observamos resultados intrigantes e inovadores:

1) O aumento de L-arginina induziu, de forma surpreendente, a produção de glutationa (GSH) pelas células-beta, além de reduzir o conteúdo de glutationa oxidada (GSSG). Isso sugere que a presença basal deste aminoácido é essencial para a síntese de GSH possivelmente por aumentar a sua conversão em glutamato, o

principal precursor de GSH (para maiores informações sobre a via metabólica sugerida, por favor, consulte o artigo 2). Sendo a GSH o principal antioxidante da célula beta, é de extrema importância que este metabólito seja formado de forma abundante e, se a arginina é capaz de induzir sua formação, não só como substrato, mas talvez por ativar enzimas do metabolismo da glutationa, sua presença constante é essencial para a manutenção da célula beta e, concordando com nossa hipótese, sua falta no microambiente da insulite pode sim ser o fator complicador no processo inflamatório que leva a morte celular e ao diabetes.

2)   O aumento de L-arginina resultou em diminuição da utilização de glutamina, aumento do consumo de glicose e redução da produção de lactato. Pela primeira vez, foi demonstramos que a L-arginina é capaz de induzir um aumento das vias aeróbias (oxidativas) e reduzir o fluxo pela via anaeróbia. Isso abre a possibilidade de outras explicações pelas quais a L-arginina é um importante secretagogo de insulina, e não somente a clássica via sugerida de influxo de cátions e despolarização celular.

3)   A célula beta apresenta uma alta atividade da enzima arginase e que esta também, assim como o caso da NOS2, pode ser ativada por citocinas pró-inflamatórias. A atividade da arginase, de forma constitutiva, parece de suma importância para uma célula que depende da formação de um radical livre (o NO) e apresenta tão baixas defesas antioxidantes contra este radical. Além disso, o aumento de sua atividade na presença das citocinas deve ocorrer de maneira a acompanhar a formação de NO a fim de reduzir a citotoxicidade mediada pro este gás. Cabe aqui, lembrar que a redução dos níveis de L-arginina e concomitante aumento de expressão de NOS2, que ocorre na insulite, pode levar a formação de peroxinitrito pela NOS2 (desacoplada), um radical muito mais citotóxico que leva a morte celular.

4)   Interessantemente, a L-arginina foi capaz de induzir a expressão de Heme-oxigenases (HO-1 e HO-3). A importância destas enzimas já fora citada no texto, mas merece atenção novamente. Os seus

produtos, monóxido de carbono (CO) e bilirrubina tem recebido atenção para seus efeitos antioxidantes, anti-inflamatórios e de manutenção da função normal de células beta. A produção de CO, especialmente, está associada a redução dos níveis de ROS, manutenção do estado redox, redução da atividade da NADPHoxidase, regulação à menor das moléculas de adesão celular dentre outros efeitos anti-inflamatórios. A ativação das HO ou da formação do CO tem sido utilizada com grande sucesso em transplantes de ilhotas além de que sua expressão constitutiva esta associada ao atraso no aparecimento e desenvolvimento do diabetes tipo I. Somado a isso, o CO foi demonstrado como um possível agente anti-citoxicidade mediada por NO uma vez que é capaz de remover o NO em processos de nitração ou nitrosilação.

5)   A secreção de insulina, na presença de níveis aumentados de L-arginina pro 24h foi levemente diminuída. Sendo a arginina um conhecido secretagogo de insulina, em concentrações mais elevadas do que a utilizada e por tempo de incubação reduzido, é possível que a presença crônica de arginina elevada tenha causado dessensibilização ao estímulo secretagogo. De fato outros aminoácidos, como a alanina, quando incubados de forma crônica tem demonstrado efeito similar.

Além do papel do exercício como modulador da mobilização de L-arginina, estudamos também, qual seria o papel da interleucina-6 sobre as células-beta, uma vez que esta citocina é a principal interleucina aumentada durante o exercício e é liberada pelo próprio músculo exercitado.

Atualmente, sabe-se que a prevenção do diabetes do tipo I requer uma intervenção direta no processo auto-imune contra as células β das ilhotas pancreáticas. Apesar do mecanismo exato desta prevenção ainda se encontrar sob investigação, nós acreditamos que o exercício, via as citocinas do tipo 2 produzidas pelos linfócitos possam modular esta resposta positiva, uma vez que elas citocinas apresentam um efeito imunossupressor. De fato, a prática de exercício físico regular e de intensidade moderada tem se mostrado um eficiente aliado no combate aos desbalanços fisiológicos

apresentados por diferentes patologias. Particularmente em relação às disfunções imunológicas, o exercício físico tem sido prescrito como uma estratégia terapêutica complementar.

A produção de citocinas, sabidamente moduladoras da resposta imune, pode ser modulada por vários fatores, como exercício físico, hormônios de estresse, baixo nível energético e estresse. Um grande número de estudos tem demonstrado que as concentrações plasmáticas de citocinas anti-inflamatórias como IL-1ra, IL-4, IL-10 aumentam após várias formas de exercício, incluindo breves sessões de exercício máximo, exercícios de força e exercícios aeróbios de longa duração (caminhada e ciclismo). O aumento da produção de citocinas antiinflamatórias durante o exercício, possivelmente sirva para conter as reações inflamatórias induzidas pelo dano muscular provocado pelo estresse físico, mas também para reduzir a produção de citocinas pró-inflamatórias associadas ao desenvolvimento de doenças como o diabetes.

Aparentemente, a produção de IL-6 pelo músculo é o principal regulador da resposta anti-inflamatória que se segue durante e após uma sessão de exercício moderado. As ações desta citocina, ou recentemente classificada, miocina, durante o exercício promove não só adaptações imunológicas, mas também se faz essencial para adaptações metabólicas necessárias para a manutenção do exercício mobilizando reservas energéticas. A maioria de suas ações, tipo lipólise no tecido adiposo e glicogenólise hepática são catabólicas, feitas com o intuito de aumentar o aporte energético para os músculos. Ao mesmo tempo, as ações anabólicas sofrem redução, para evitar ciclos metabólicos fúteis. Independente da ação da IL-6, todas elas tem sido associadas benéficas em favor a melhora imunológica e metabólica, especialmente para o tratamento do diabetes. Apesar disso, vários estudos têm associado os níveis elevados de IL-6 com o desenvolvimento de patologias tais como obesidade, aterosclerose, diabetes tipo II, entre outros. Muitos destes estudos mostraram uma alta associação entre a doença e os níveis de IL-6. No entanto, esta citocina pode estar aumentada em uma condição patológica e não ser a causa, mas sim uma conseqüência da perturbação fisiológica, e pode apresentar, na verdade, uma função de regular-à-menor a disfunção orgânica.

Por esta razão, investigamos o efeito exclusivo da IL-6 sobre a secreção de insulina, metabolismo, sinalização celular e estado redox celular de uma célula clonal secretora de insulina (BRIN-BD11), utilizando concentrações de IL-6 similares as

encontradas durante o exercício moderado (50μg/mL). Observamos, após as incubações com IL-6, interessantes resultados:

1) A IL-6 causou um aumento significativo nas secreções crônica e aguda de insulina, bem como um aumento do consumo de glicose pelas células beta. Este trabalho demonstra, pela primeira vez, uma possível conversa entre o músculo esquelético, produtor e liberador de IL-6, com as células-beta do pâncreas mediado por esta miocina.

2) A IL-6 causou um aumento no conteúdo da forma ativa da AMPK, sua forma fosforilada (AMPK-p) com concomitante aumento na expressão de NOS2 e formação de nitritos. Indicando que estas vias, por suas ações metabólicas, podem estar envolvidas no processo de aumento do consumo de glicose e secreção de insulina, uma vez que própria AMPK é uma enzima chave no metabolismo de ácidos graxos. De forma interessante, estas vias parecem estar relacionadas e serem dependentes, uma vez que a ativação da AMPK já foi associada, em outras células, com a ativação da NOS2 e de seus produtos (para maiores informações sobre esta relação, por favor, consulte a discussão do estudo 1.

Sabendo que durante o exercício a secreção de insulina é reduzida e que isto é essencial para evitar fluxos metabólicos fúteis durante o exercício (e, é claro, para a manutenção da glicemia), pode parecer estranho, a primeira vista, qual a razão de uma miocina que é liberada pelo músculo durante o exercício e tem por função a mobilização de substratos para a continuação da atividade, exercer uma função positiva sobre a secreção de insulina. Conforme sugerido pelos autores no estudo 1, esta ação esta relacionada com a fase de recuperação que segue após o exercício. Ou seja, durante o exercício, a IL-6 e as catecolaminas têm como função a mobilização das reservas energéticas para a execução do exercício. Neste momento, a secreção de insulina é inibida pela ação das catecolaminas. Finalmente, ao término da atividade, inicia-se a fase de recuperação, onde os níveis de insulina rapidamente aumentam e a captação de glicose e aminoácidos também, com objetivo de síntese e adaptação. Este aumento de insulina, antes atribuído a redução abrupta de adrenalina, agora também pode ser associado ao nível aumentado de IL-6 ao final do exercício.

Interessantemente, a IL-6 aparentemente pode, a longo prazo, induzir outros efeitos anabólicos, como por exemplo aumentar a sensibilidade de vias sabidamente anabólicas como as ativadas pela mTOR no músculo esquelético. Além disso, a IL-6 é capaz de reduzir a produção de TNF-α, uma citocina sabidamente associada com proteólise e com resistência a insulina. Finalmente, estas ações da IL-6 sobre a célula beta e sobre as células musculares, levam claramente a apontar o exercício físico como ferramenta para o tratamento, e talvez prevenção do diabetes pela sua ação positiva aumentando a sensibilidade das células beta e reduzindo a resistência à insulina, todos os efeitos mediados por esta miocina.

Para finalizar, podemos sugerir que o exercício físico, como modulador da liberação de citocinas e da mobilização de L-arginina/glutamina, é uma ferramenta á ser considerada no tratamento e prevenção do diabetes. Estudos *in vivo* sobre esta hipótese estão sendo testados em nosso laboratório e em parceria com o laboratório de metabolismo da University College Dublin (UCD).

ANEXOS

CATALOGAÇÃO NA FONTE