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A Novel L-Arginine/L-Glutamine Coupling Hypothesis: Implications for Type 1 Diabetes

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1. Introduction

L-Arginine is synthesised *in vivo* from L-glutamine, L-glutamate, or L-proline via the intestinal-renal axis (**Fig. 1A**) in humans and most other mammals (Wu et al., 2009). In humans, plasma L-glutamine is the precursor of 80% of plasma L-citrulline while plasma L-citrulline, in turn, is the precursor of 10% of plasma L-arginine (van de Poll et al., 2007). Although the intestine consumes L-glutamine at a high rates, dependent on L-glutamine supply (and production from the skeletal muscle), approximately 13% of L-glutamine taken up by the intestine is converted to L-citrulline, so that quantitatively, L-glutamine is the major precursor for intestinal release of L-citrulline (van de Poll et al., 2007), which can be further converted to L-arginine. These observations highlight the importance of L-arginine/L-glutamine metabolic coupling, especially as L-arginine is one of the most potent secretagogues of insulin from the pancreatic beta cells (Palmer et al., 1976), whereas L-arginine deficiency is associated with insulinopenia and failure to secrete insulin in response to glucose (Spinas et al., 1999). L-Arginine is essential for metabolism and function of multiple body organs, with decreased plasma and cellular levels of L-arginine reported in type 2 diabetic subjects (Pieper & Dondlinger, 1997).

Since L-arginine is the precursor of nitric oxide (NO)*, which serves as a key cell signalling molecule in pancreatic islet β -cells, restriction in the availability of L-arginine is likely to

* **Abbreviations used:** CAT, catalase; GSH, glutathione; GSSG, glutathione disulphide; GSPx, glutathione peroxidase; GSRd, glutathione disulphide reductase; HSP70, 70-kDa member of heat shock protein family; eHSP70, extracellular heat shock protein of 70 kDa; IFN- γ , interferon- γ ; I κ B, a member of the inhibitors of nuclear factor κ B; IKK, inhibitor of κ B kinase; IL-1 β , interleukin-1 β ; IL1-ra, IL-1 β receptor antagonist; iNOS, inducible nitric oxide synthase; NF- κ B, a member of nuclear transcription factor κ B; NO, nitric oxide free radical (•N=O); PPAR- γ , peroxisome proliferator activated receptor- γ ; RNS, reactive nitrogen species; ROS, reactive oxygen species; SNOG, S-nitrosoglutathione; SOD,

mechanical activity, where blood glutamine stores may be challenged. Metabolic acidosis, by increasing L-glutamine utilisation 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 (**Fig. 3**), which ultimately leads to a Th1-centered immune response that augments glutamine utilisation. Therefore, L-glutamine imbalance, by virtue of deficiently supplying L-arginine to the pancreas, deviates β -cell glutamate metabolism from the synthesis of GSH to that of NO, leading to oxidative stress, impairment of insulin release and insulinitis. This ongoing inflammation feeds forward NO metabolism, which enhances L-glutamine consumption thus perpetuating this cyclic condition that leads to type 1 diabetes mellitus (T1DM) (**B**). Physical exercise, on the other hand, may improve L-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. Arrow widths indicate the intensity of the metabolic flux through each pathway.

contribute to derangements in the secretion and action of insulin (Newsholme et al., 2009a). Hypertension associated with diabetes is related with a decrease in levels of L-arginine (Spinass, 1999), as are inflammatory conditions characterised by release of L-arginase by activated macrophages (Murphy & Newsholme, 1998). While excessive NO production can trigger oxidative/nitrosative stress and is undoubtedly a key mechanism that results in β -cell death (Newsholme et al., 2009a; Spinass, 1999; Michalska et al., 2010), good evidence now suggests that lesser amounts of cellular NO, produced by the NF- κ B-regulated inducible nitric oxide synthase (iNOS, EC 1.14.13.39), encoded by the *NOS-2* gene, serves as an important coupling factor in insulin secreting cells (Newsholme et al., 2009a; Spinass, 1999; Michalska et al., 2010). Recent data from the authors' laboratories has demonstrated that L-arginine is an important stimulator of β -cell glucose consumption and intermediary metabolism (M.S. Krause, N.H. McClenaghan, P.R. Flatt, P.I. Homem de Bittencourt Jr., C. Murphy & P. Newsholme, unpublished results). Such actions lead to increased insulin secretion, enhanced antioxidant and protective responses with greater functional integrity when challenged with pro-inflammatory cytokines. Given that insulin-secreting cells have very low expression levels of antioxidant enzymes, such as catalase (CAT) and glutathione peroxidase (GSPx), β -cells are particularly prone to chemical stress in the diabetogenic or inflammatory environment typical of type 1 and possibly type 2 diabetes (Newsholme et al., 2009a; Spinass, 1999). In fact, the pathogenesis of type 2 diabetes is now recognised to involve both innate and adaptive immunity, since type 2 diabetes is associated with low-grade systemic inflammation, infiltration of adipose tissue and pancreatic islets with CD8⁺ T lymphocytes that precede invasion by inflammatory macrophages and activation of these cells resulting in pro-inflammatory cytokine secretion (Mandrup-Poulsen, 2010).

In this chapter, we discuss how the continued supply of L-arginine, physiologically provided by the metabolism of L-glutamine via the intestinal-renal axis and from active skeletal muscle (which will be enhanced during exercise) is essential for β -cell functional integrity and indeed for β -cell defence, which will be required to avoid/attenuate islet inflammation associated with the pathogenic mechanisms underlying type 1 and type 2 diabetes (**Fig. 1B**). L-arginine is therefore preserved for essential NO generation and stimulation of glucose metabolism, critical for insulin secretion. Additionally, the role of skeletal muscle (during exercise) on these metabolic processes is discussed.

2. Oxidative metabolism and oxidative stress in β -cells and type 1 diabetes

The intense aerobic metabolism, intrinsic to pancreatic β -cells, exposes these cells to the deleterious effects of high-turnover oxygen-based reactions. In fact, during secretagogue-stimulated insulin secretion, β -cells are associated with accelerated mitochondrial flux of electrons and, consequently, elevated tendency towards reactive oxygen species (ROS) production (Newsholme et al., 2007). However and notably, β -cells present a very low level of expression of antioxidant enzymes such as CAT and GSPx compared with other tissues and this reduced antioxidant activity is associated with significant increases in lipid hydroperoxides, conjugated dienes and protein carbonyls, which are markers for oxidative stress (Santini et al., 1997), so that β -cells are intrinsically prone to oxidative stress.

Moreover, a growing body of evidence indicates that, in the pre-diabetic condition, antioxidant status may be impaired (Rocic et al., 1997). 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 categorises the onset of type 1 and type 2 diabetes.

Oxidative stress has long been recognised to play an important role in the development of type 1 diabetes and its subsequent complications (Wierusz-Wysock et al., 1997) which are aggravated due to the low activities of oxygen free radical scavenging enzymes in islet β -cells, especially mitochondrial manganese-type superoxide dismutase (Mn-SOD; Asayama et al., 1986), glutathione peroxidase (GSPx; Malaisse et al., 1982; Mathews & Leiter, 1999) and glutathione disulphide (GSSG) reductase (GSRd; Mathews & Leiter, 1999). 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 (Lenzen et al., 1996). Additionally, the administration of antioxidants (nicotinamide, SOD, α -tocopherol, probucol and the 21-aminosteroid lazaroids), as well as oxygen free radical scavengers, have been used *in vitro* to protect islets from the cytotoxic effects of some pro-inflammatory cytokines (IL-1 β , TNF α and IFN γ), concurrently providing *in vivo* protection against the development of the autoimmune diabetes process (Nomikos et al., 1986). Conversely, studies on MnSOD and CAT transgenics have shown that protection of islets from oxidative stress does not alter cytokine toxicity (Chen et al., 2005), 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.

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 O₂⁻ generation. Since O₂⁻ anion is a powerful activator of UCP2, a positive feedback mechanism exists in that O₂⁻ generation enhances its own formation. This is particularly critical under prolonged hyperglycaemia, where UCP2 activity may be extremely high thus further depressing insulin secretion by β -cells (Newsholme et al., 2007). This situation is probably associated with the development of type 2 diabetes. 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 (Morgan et al., 2007, Newsholme et al., 2009b).

Type 1 diabetic patients 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 these patients is typically observed (Maxwell et al., 1997). Diabetic children show significant reduction in GSH and GSPx in erythrocytes, as well as in plasma α -tocopherol and β -carotene levels (Dominguez et al., 1998). Incubation of rat (Rabinovitch et al., 1992) and human (Rabinovitch et al., 1996) islet cells with a cytotoxic combination of cytokines (IL-1 β , 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 cytotoxic effects in rat islets (Sumoski et al., 1989). Taken together, these findings suggest that cytokine-induced inhibition of insulin release may not be oxygen free radical-mediated, whereas the cytotoxic effects of cytokines on β -cells do appear to involve free radical-mediated events that induce the formation of toxic derivatives within the islets of Langerhans (Suarez-Pinz et al., 1996). This strongly suggests that type 1 cytokines interfere in β -cell metabolism at some point that is intimately related to insulin secretion. But where does reside this extreme sensitivity of β -cells to cytokine signals? The expression of iNOS, necessary for the synthesis of NO during insulin secretion, may provide an explanation.

NO has incontestably been shown to be a physiological regulator of insulin secretion in β -cells, in an elegant experimental protocol designed by Prof. Anne Marie Salapatek's group in Canada and reported in a seminal paper (Smukler et al., 2002). 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 reported that NO donation did not elicit a β -cell intracellular Ca^{2+} ($[Ca^{2+}]_i$) response alone, but was able to potentiate a glucose-induced $[Ca^{2+}]_i$ response. Since NO is a strong 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 (Spinas, 1999). Therefore, overproduction of NO related to inflammatory stimuli may be related to cellular dysfunction but **not** normal levels of NO. As previously argued (Smukler et al., 2002), 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 (Moncada et al., 1991; Beck et al., 1999). Hence, the supraphysiological elevation of L-arginine, or the application of exogenous NO donors under the condition of already elevated NO, may result in excessive NO production, yielding cytotoxic effects (Smukler et al., 2002).

3. Nuclear factor κ B-dependent L-arginine metabolism in β -cells

Pancreatic β -cells have to constantly express NF- κ B-regulated iNOS in order to achieve appropriate amounts of NO produced from L-arginine. However, inflammatory cytokines, such as IL-1 β and TNF- α , activate NF- κ B in rodent and human islet cells (Eizirik & Mandrup-Poulsen, 2001). Contrarily, prevention of NF- κ B activation protects pancreatic β -cells against cytokine-induced apoptosis (Giannoukakis et al., 2000; Heimberg et al., 2001). It is impressive that about 70 NF- κ B-dependent genes have been currently identified in β -cells, including genes encoding for various inflammatory cytokines and iNOS (Darville & Eizirik, 1998). 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 (Kutlu et al., 2003). It is of note that treatment of human, as well as rodent β -cells with purified IL-1 β alone is not sufficient to induce apoptosis, but if IL-1 β is combined with interferon- γ (IFN γ), β -cells undergo apoptosis after few days in culture (Eizirik & Mandrup-Poulsen, 2001). This suggests that an intracellular IFN γ signal must synergise with IL-1 β signalling pathways in order to trigger β -cell apoptosis. 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 dimerises and translocates to the nucleus where binding to γ -activated sites on target genes occurs (Eizirik & Mandrup-Poulsen, 2001). STAT-1 mediates the potentiating effect of IFN γ on IL-1 β -induced iNOS expression (Darville & Eizirik, 1998). 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 (Karlsen et al., 2001; Flodstrom et al., 2003). SOCS-3 also protects insulin-producing cells against IL-1 β -mediated apoptosis via NF- κ B inhibition (Karlsen et al., 2004). 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.

Besides its activation by cytokines, NF- κ B is also a potential target for reactive oxygen/nitrogen species (ROS/RNS). It is noteworthy that NF- κ B was the first redox-sensitive eukaryotic transcription factor shown to respond directly to oxidative stress in many types of cells (Dröge, 2002), while its activation leads to the expression of at least a hundred of inducible proteins directly involved in inflammation, such as cyclooxygenase-2 (COX-2), iNOS, TNF α and IL-1 β (Moynagh, 2005). Therefore, NF- κ B is, at the same time, both a target and an inducer of inflammation and inflammation-induced oxidative stress. In resting (unstimulated) cells, NF- κ B dimeric complexes are predominantly found in the cytosol where they are associated with members of the inhibitory I κ B family (Moynagh, 2005), so that NF- κ B gene products are entirely inducible proteins whose activation is dictated by specific stimuli that activate I κ B kinase (IKK) complexes. These stimuli include high intracellular GSSG levels and oxidative stress *per se* (Dröge, 2002). IKKs, in turn, phosphorylate I κ B proteins directing them to proteasome-mediated degradation, which sets NF- κ B dimers free to bind to DNA in the nucleus.

NF- κ B activation is responsible for both initiation and amplification of immune and inflammatory responses in all cells. Actually, NF- κ B activation is *sine qua non* for the control of immune and inflammatory responses (Baldwin, 1996; Nakamura et al., 1997; Winyard et al., 1997), and since inflammatory factors, such as pro-inflammatory cytokines, chemokines, adhesion molecules, colony-stimulating factors and inflammatory enzymes, are NF- κ B-dependent gene products, dysregulation or aberrant activation of NF- κ B could initiate inappropriate autoimmune and inflammatory responses. Conversely, inhibition of NF- κ B activation has been argued as a potential therapeutic approach in several immune and inflammatory-related diseases (Chen et al., 1999). This is why cyclopentenone prostaglandins (cp-PGs), which are powerful inhibitors of NF- κ B activation (Rossi et al., 2000), are now considered to be the physiological mediators of the “**resolution of inflammation**” (Piva et al., 2005), whereas cp-PG-based pharmacological approaches, *e.g.* LipoCardium technology, which is a liposome contained cp-PG-based formulation specifically directed towards atherosclerotic lesions in arterial walls (Homem de Bittencourt et al., 2007; Gutierrez et al., 2008) have proved to be powerful anti-atherosclerotic strategies (Piva et al., 2005; Ianaro et al., 2003; Homem de Bittencourt Jr., 2007).

Finally, considering that all the known forms of inflammation finish with the formation of naturally-occurring anti-inflammatory agents (*e.g.* cp-PGs, IL-10), an important question remains as to how does β -cell not resolve inflammation by triggering such responses? A fault in the expression of the anti-inflammatory heat shock proteins may give a clue to this question.

4. Heat shock protein pathways

Heat shock proteins (HSPs) have been found to play a fundamental role in the recovery from multiple stress conditions and to offer protection from subsequent insults (De Maio,

2011). The function of HSPs during stress goes beyond their intracellular localization and chaperone role as they have been detected outside cells activating signaling pathways. Extracellular HSPs are likely to act as indicators of the stress conditions, priming other cells, particularly of the immune system, to avoid the propagation of the insult (see De Maio, 2011 for review). As we shall present below, the delicate balance between the “danger signalling” extracellular HSPs and its intracellular counterparts may dictate pancreatic β -cell response to cytokines and, eventually, the precipitation of diabetes. By regulating L-arginine consumption through iNOS, and, consequently, NO generation, intracellular HSP response (or its deficiency) may unravel unpredicted facets of both type 1 and type 2 diabetes.

Heat shock proteins (HSPs) are a set of highly conserved polypeptides in both eukaryotic and prokaryotic organisms. They are categorised in families according to their molecular sizes and include HSP110, HSP100, HSP90, HSP70, HSP60 HSP30 and HSP10 subclasses. By far, the most studied (due to its evident high expression in mammalian cells under stress conditions) and conserved is the 70-kDa family (HSP70), which comprises a number of related proteins whose molecular weights range from 66 to 78 kDa. HSP70 isoforms are encoded by a multigene family consisting presently of, at least, 13 distinct genes in humans so far studied (Kampinga et al., 2009; Henderson, 2010). Human HSP70 is 73% identical to *Drosophila* HSP70 and 47% identical to *E. coli* DnaK (the *E. coli* orthologue of eukaryotic HSP70) while, surprisingly, the nucleotide sequences of the human and *Drosophila* genes are 72% identical and human and *E. coli* genes are 50% identical (Hunt & Morimoto, 1985). HSP70s function as molecular chaperones that facilitate protein transport, prevent protein aggregation during folding, and protect newly synthesised polypeptide chains against misfolding and protein denaturation (Henderson, 2010). While the constitutive form is expressed in a wide variety of cell types at basal levels (being only moderately inducible), the so-called inducible HSP70 forms (which are barely detectable under non-stressful conditions) could be promptly synthesised under a condition of “homeostatic stress”, this being any “homeostasis threatening” condition, such as heat, glucose deprivation, lack of growth factors and so forth. Traditionally, research groups indistinctly use HSP70 as a unified term for both inducible (72 kDa, HSP72 encoded by the *HSPA1A* human gene) and constitutive (73 kDa, HSP73 or HSC70, for heat shock cognate protein, encoded by the human *HSPA8* gene whose product differs from *HSPA1A* protein by only 2 amino acids, Kampinga et al., 2009; Tavaría et al., 1996; Arya et al., 2007; Tavaría et al., 1995). However, HSP70 is the preferable form to be used only when one refers to the inducible HSP72 protein encoded by *HSPA1A* gene (Heck et al., 2011).

Many different events can induce HSP expression, among them are environmental, pathological and physiological factors, such as heavy metal exposure, UV radiation, amino acid analogues, bacterial or viral infection, inflammation, cyclo-oxygenase inhibitors (including acetylsalicylic acid), oxidative stress, cytostatic drugs, growth factors, cell differentiation and tissue development, which strongly activate the main eukaryotic heat shock transcription factor, HSF-1, leading to HSP70 expression (Lindquist & Craig, 1988). Physical exercise, even at single low-intensity bouts (Silveira et al., 2007), is able to induce HSP70 expression in different cell types leading to augmented plasma HSP70 concentrations (see Heck et al., 2011 for review). In our hands, rats submitted to swimming sessions of as short as 20 min (2-4% body weight overload, a mild exercise) demonstrate increased HSP72 (mRNA and protein) in circulating monocytes and lymphocytes and in lymph node lymphocytes and peritoneal macrophages, which is paralleled by a rise in plasma HSP70 levels immediately after the exercise (C.M. Schöler, S.P. Scomazzon, P. Renck Nunes, T.G. Heck, P.I. Homem de Bittencourt Jr., unpublished work).

4.1 Intracellular hsp70

Aside the now classical molecular chaperone action, the most remarkable intracellular effect of HSP70s is the inhibition of NF- κ B activation, which has profound implications for immunity, inflammation, cell survival and apoptosis. Indeed, HSP70 blocks NF- κ B activation at different levels, by inhibiting the phosphorylation of the inhibitor of κ B (I κ Bs), by directly binding to I κ B kinase- γ (IKK γ) thus inhibiting tumour necrosis factor- α (TNF α)-induced apoptosis (Ran et al., 2004). In fact, the supposition that HSP70 might act intracellularly as a suppressor of NF- κ B pathways has been raised after a number of discoveries in which HSP70 was intentionally induced, such as the suppression of astroglial iNOS expression paralleled by decreased NF- κ B activation (Feinstein et al., 1996) and the protection of rat hepatocytes from TNF α -induced apoptosis by treating cells with the NO-donor S-Nitroso-N-acetylpenicillamine (SNAP), which reacts with intracellular glutathione (GSH) molecules generating S-nitrosoglutathione (SNOG) that induces HSP70, and, consequently, HSP70 expression (Kim et al., 1997).

HSP70 confers protection against sepsis-related circulatory mortality via the inhibition of iNOS gene expression in the rostral ventrolateral medulla through the prevention of NF- κ B activation, inhibition of I κ B kinase activation and consequent inhibition of I κ B degradation (Chan et al., 2004). This is corroborated by the finding that HSP72 assembles with hepatocyte NF- κ B/I κ B complex in the cytosol thus impeding further transcription of NF- κ B-depending TNF- α and NOS-2 genes that would worsen sepsis in rats (Chen et al., 2005). This may also be unequivocally demonstrated by treating cells or tissues with HSP70 antisense oligonucleotides that completely reverses the beneficial NF- κ B-inhibiting effect of heat shock and inducible HSP70 expression (see, for instance, Kim et al., 1997; Chan et al., 2004). Hence, HSP70 is anti-inflammatory *per se*, when intracellularly located, which also explains why cyclopentenone prostaglandins (cp-PGs) are powerful anti-inflammatory autacoids (Rossi et al., 2000; Homem de Bittencourt & Curi, 2001; Beere, 2004; Gutierrez et al., 2008).

Another striking effect of HSP70 is the inhibition of apoptosis, which occurs via many intracellular downstream pathways (e.g. JNK, NF- κ B and Akt) that are both directly and indirectly blocked by HSP70, besides the inhibition of Bcl-2 release from mitochondria (Beere, 2004). Therefore, intracellularly activated HSP70s are cytoprotective and anti-inflammatory by avoiding protein denaturation and excessive NF- κ B activation which may be damaging to the cells.

It is strikingly noteworthy that L-glutamine attenuates TNF- α release and enhances HSP72 expression in human peripheral blood mononuclear cells (Wischmeyer et al., 2003). In fact, L-glutamine induces HSP70 expression via O-glycosylation and phosphorylation of HSF-1 and Sp1 (Singleton, K.D. & Wischmeyer, P.E., 2008) in a process that is mediated, at least partially, by the increase in the flux through the hexosamine biosynthetic pathway (Hamiel et al., 2009). Also, it has been shown that a single dose of L-glutamine relieve renal ischaemia-reperfusion injury in rats in 24 h by a mechanism associated with enhanced HSP70 expression (Zhang et al., 2009).

4.2 Extracellular hsp70

HSP70s may also be found in the circulation and its presence is associated to oxidative stress. While healthy people usually have low plasma levels of HSP70, the association of increased blood concentrations of such proteins with illness and disease progression has been hypothesised. In this way, oxidative stress, inflammation, cardiovascular disorders and

pulmonary fibrosis have been directly correlated with HSP70 concentration in the bloodstream (Ogawa et al., 2008). On the other hand, L-glutamine supplementation, which rises circulating HSP70 levels in critically ill patients, is associated with lower hospital treatment period (Ziegler et al., 2005). Therefore, these studies may suggest that elevation of HSP70 levels could be an important immunoinflammatory response against physiological disorders or disease.

Inasmuch as HSP70s exist in the extracellular space, molecular interactions with cell surface receptors may occur and signalling pathways could be triggered in many cell types, whereas there are a variety of receptors to HSP70 binding, amplifying the possible targets to these extracellular molecules (Calderwood et al., 2007a, 2007b). However, the function of circulating HSP70 is incompletely understood yet. HSP70s are released towards the extracellular space by special mechanisms that include pumping across cell membranes through the highly conserved ABC cassette transport proteins. Recent studies have demonstrated that exosomes provide the major pathway for the vesicular secretory release of HSP70s and that heat stress strikingly enhances the amount of HSP70 secreted per vesicle, but does not influence the efficiency of stress-induced rate of HSP70 release and the number of exosomes neither (Sun et al., 2005; Lancaster & Febbraio, 2005; Multhoff, 2007). A similar profile was observed in our hands (T.G. Heck; P. Renck Nunes; S.P. Scomazzon & P.I. Homem de Bittencourt Jr., manuscript in preparation), in which lymph node lymphocytes from exercised rats submitted to a further (other than the exercise bouts) challenge (heat shock) presented an HSP70 accumulation into the culture medium that is dependent on previous exercise load. Apparently, systemic extracellular HSP70 (eHSP70) could arise from many tissues and different cell types and this may involve distinct mechanisms of release (including necrosis) and a large variety of inducing factors (Mambula et al., 2007). Finally, HSP72 is clearly the major component of the secreted eHSP70 found in the circulation, although recent evidence suggests that other forms may also be released into the blood, as recently pointed out by De Maio (2011). eHSP70 has been shown to bind to type 2 and 4 toll-like receptors (TLR2 and TLR4) on the surface of antigen-presenting cells (APCs) similarly to lipopolysaccharides (LPS), inducing the production of the pro-inflammatory cytokines IL-1 β and TNF- α , as well as NO (a product with prominent anti-microbial activity), in an NF- κ B-dependent fashion (Ao et al., 2009; Asea, 2003; Asea, 2008).

Taken together, the above findings suggest that the body must attain a precise equilibrium between pro-inflammatory eHSP70 and anti-inflammatory intracellular HSP70 production in order to avoid chronic non-resolved inflammations, such as those observed in sepsis and during the onset of type 1 diabetes. However, why such a balance is not achieved in these illnesses is a matter of intense study.

4.3 Heat shock proteins and exercise

As recently reviewed (Heck et al., 2011), physical exercise and its inherent physiological alterations induce HSP70 expression in many tissues and cell types, not only in the muscle cells. The breakdown of cell homeostasis produced by modifications in temperature, pH, ion concentrations, oxygen partial pressure, glycogen/glucose availability, and ATP depletion are among the factors that activate HSP70 synthesis during exercise (Noble et al., 2008). Rise in core and muscle temperature during exercise seems an obvious way to induce HSP70. However, while skeletal muscle sustains HSP70 expression in the absence of heat stimulus,

the heart is not able to do the same, thus suggesting that the mechanisms of HSP70 protein synthesis are specifically driven in each tissue (Harris & Starnes, 2001; Skidmore et al., 2005; Morton et al., 2007; Staib et al., 2007) and that augmented temperature is insufficient to elicit HSP70 synthesis during exercise. Moreover, the susceptibility of tissues to be stressed by the environmental changes elicited by exercise varies enormously and other protective pathways may be activated in the heart, as we have shown for MRP/GS-X pump ATPases whose expression seems to prevent HSP70 expression in the cardiac muscle after exercise bouts (Krause et al., 2007). In spite of free radicals may be produced under normal conditions, a burst in reactive oxygen species does occur during exercise (Fisher-Wellman & Bloomer, 2009). Besides enzymatic and non-enzymatic antioxidant apparatus, studies in both animal models and humans implicate HSP70s as a complementary protection against oxidative damage (Smolka et al., 2000; Silmar et al., 2007; Hamilton et al., 2003), particularly because HSP70s may recover oxidatively denatured proteins. After an acute exercise session, skeletal muscle (Hernando & Manso, 1997), cardiac muscle (Locke et al., 1995) and other tissues, such as the liver (Gonzalez & Manso, 2004; Kregel & Moseley, 1996), have shown a state of oxidative stress, concomitantly to high concentrations of intracellular HSP70 (Salo et al., 1991). Even though oxidative stress is a strong factor to induce HSP70s in response to exercise, free radical production is not the only pathway involved in this process, since sexual hormones and adrenergic stimuli may modulate HSP70 response (Parro & Noble, 1999; Parro et al., 2002a, 2002b; Parro et al., 1999) and circulating monocytes from acutely exercised rats do not show appreciable changes in erythrocyte glutathione disulphide (GSSG) to glutathione (GSH) ratio (an index of intracellular redox status) and plasma thiobarbituric acid-reactive substances (TBARS), even in a state of high-profile synthesis of hydrogen peroxide (Silveira et al., 2007).

More recently, however, it has been demonstrated the presence of HSP70s in the circulation in response to exercise (Walsh et al., 2001). Since exercise is able to induce high concentrations of HSP70s in both muscle and plasma, the most obvious hypothesis was, primarily, that skeletal muscle should be the releaser of HSP70 during exercise. However, further studies have revealed that this is not the case, at all. Postural muscles express high levels of HSP70s under basal conditions, which has led to the belief in a preventive role for these proteins against muscle damage through the stabilization of ionic channels (Tupling et al., 2007), as well as myotube development (Kayani et al., 2008). HSP70s were also believed to be an important way to preserve low twitch (oxidative) muscle phenotype after frequent activation, as in physical training (Kelly et al., 1996; Murlasits et al., 2006). Preservation of intracellular muscular function during different exercises, venous-arterial HSP70 differences in different territories (Febbraio et al., 2002a), and the lack of evidence supporting the proposition that the muscle could be the major source of circulatory eHSP70 precluded the 'muscle hypothesis' and suggested that other tissues/cells should be responsible for the increase of eHSP70 in the circulation. Once HSP70 protein release from the muscle to the extracellular fluid could eventually happen by lysis process, and considering that the lysis of muscle fibre occurs only under severe cellular stress condition, the presence of eHSP70 during moderate exercise, as we normally employ, was found to be unfeasible. Though it had been shown that both the intensity and duration of exercise have effects in plasma eHSP70 (Fehrenbach et al., 2005) and muscle (Milne & Noble, 2002) HSP70 immunoccontents, this rise in circulating levels of eHSP70 precedes, however, any gene or protein expression

of HSP70 in skeletal muscle (Febbraio et al., 2002b), which is another strong argument against the 'muscle hypothesis'. As stated above, other tissues synthesise HSP70s during physiological challenges to the homeostasis, as in an acute physical exercise bout. In this way, after treadmill exercise protocol, the rat liver has been found to enhance the expression of HSP70s (Gonzalez & Manso, 2004). Moreover, and finally, in a human study featuring leg and hepatosplanchnic venous-arterial eHSP70 difference in response to exercise it was unequivocally demonstrated that the contracting muscle does not contribute to eHSP70 circulating levels, while hepatosplanchnic viscera release eHSP70 from undetectable levels at rest to 5.2 pg/min after 120 min of exercise (Febbraio et al., 2002a). Additional studies have shown that oral glucose administration may exclusively reduce HSP70 release from the liver without any effect on muscle glycogen content or intracellular expression of HSP70 (Febbraio et al., 2004). Taken together, these results suggest that other cells may release eHSP70 during exercise, as verified during an experiment that analysed cerebral venous-arterial HSP70 difference (Lancaster et al., 2004). Although the liver seems to participate in this process, the nature of eHSP70-releasing cell(s) during exercise remains to be established.

4.4 HSP70 and glucose/insulin status

Intracellular HSP70 expression produces a clear anti-inflammatory effect by knocking down the expression of pro-inflammatory NF- κ B-dependent pathways. However, the activation of HSP70 pathways produces a much more delicate effect. Accordingly, in obese insulin-resistant mice, chronic heat shock treatment has been shown to dramatically reduce insulin resistance by HSP72-specific prevention of c-Jun N-terminal Kinase (JNK) phosphorylation, an effect which is also observed in high-fat fed HSP72^{+/+} transgenic mice (Chung et al., 2008). Also, elevated expression of HSP70 has also been found in circulating mononuclear cells from type 2 diabetic patients (Yabunaka et al., 1995), which, as discussed above, is a immunoinflammatory disease as well. On the other hand, in rat islets, L-glutamine, which is an activator of HSF-1, was shown to attenuate ischaemic injury through the induction of HSP70 (Jang et al., 2008). Moreover, the well known inhibitory effect of IL-1 β and TNF- α (alone or combined) on insulin secretion may be completely prevented by a 1-h heat shock (42°C) pre-treatment of both human and rat islets (Scarim et al., 1998). These authors have also shown that the protective effects of heat shock on islet metabolic function are associated with the inhibition of IL-1 β - and TNF α -stimulated NF- κ B nuclear localization and the consequent iNOS expression. Conversely, NO was found to be one of the triggers of HSP70 expression in human islets (Scarim et al., 1998), which is similar to that previously encountered by Kim et al. (1997), who described a protective effect of NO (via the formation of SNOG that induces HSP70) in rat hepatocytes against TNF α -induced apoptosis. Moreover, J-type cyclopentenone prostaglandins (cp-PGs), which are the most powerful anti-inflammatory substances ever known (see Gutierrez et al., 2008 for review) and natural ligands of peroxisome-proliferator activated receptor- γ (PPAR- γ ; Forman et al., 1995; Kliewer et al., 1995), are the strongest inducers of HSP70 expression and consequent NF- κ B blockade, a pattern that is shared with synthetic antidiabetic thiazolidinediones (TZDs), such as rosiglitazone, pioglitazone, troglitazone, and ciglitazone (see Zingarelli & Cook, 2005, for review).

The above observations point out again to the importance of poised L-arginine-dependent NO production by β -cells in order to achieve an optimum of HSP70 expression, which may,

in turn, allow iNOS expression (needed to NO-assisted insulin secretion) but not at exaggerated ratios that culminate with β -cell death and failure in insulin secretion. In fact, physical exercise, which may also present an anti-inflammatory effect by virtue of its ability to induce the expression of HSP70, is inversely associated with L-arginine utilisation by β -cell iNOS (Atalay et al., 2004). Furthermore, a dramatic scenario does exist in that the susceptibility to oxidative damage to β -cells in type 1 diabetes is associated to the impairment of HSP70-induced cytoprotection, while endurance training may offset some of the adverse effects of diabetes by upregulating tissue HSP70 expression (Atalay et al., 2004). Indeed, in many, if not all, severe inflammatory manifestations of acute nature, such as sepsis or insulinitis, the stage of HSP70-based “resolution of inflammation” is simply not seen at all. For instance, in the serum of septic patients with highly oxidative profile (whose prognosis is death), it is observed 30-fold increase in serum HSP70 (eHSP70) compared with control subjects (Gelain et al., 2011), whereas the amount of intracellular HSP70 expressed in the cells of such subjects is, as a rule, lower than that expected. Corroborating this proposition, the expression of HSP70 by pancreatic islets from diabetes-prone BB rats has been found to be lower than that in diabetic-resistant LEW rats of same age and, in the diabetes-prone BB rats, HSP70 expression has shown to be much lower in young as compared to adult animals (Wachlin et al., 2002). Since intracellular HSP70 functions as a potent anti-inflammatory cellular tool due to the impairment over NF- κ B downstream pathways, a deficient HSP70 may threaten β -cell survival (see Hooper & Hooper, 2005, for review).

Results from our group have also shown that, besides a reduction in peripheral insulin resistance, heat shock treatment (which also enhances HSP70 export towards the plasma) may impair insulin action under hypoglycaemic conditions in the rat model (M.S. Ludwig; V.C. Mingueti; P. Renck Nunes; T.G. Heck; R.B. Bazotte & Homem de Bittencourt, P.I. Jr., manuscript in preparation) so that HSP70 balance seems to be crucial for glucose-insulin homeostasis. Now, we are currently evaluating the possibility that exercise may stimulate Th2-based immune response and protect β -cells from pro-inflammatory cytokine pathways through HSP70 induction, which, ultimately, may prevent type 1 diabetes. Since **a)** L-glutamine is a major precursor of L-arginine, which is capital for β -cell survival, **b)** L-arginine-dependent moderate NO synthesis induces HSP70 and **c)** physical exercise is able of directly inducing HSP70 and of enhancing L-glutamine production by the skeletal muscle, both exercise and/or L-glutamine supplementation are argued as preventive agents against the installation of type 1 diabetes by re-establishing the HSP70 equilibrium between the intra and extracellular spaces, as previously hypothesised (Krause & Homem de Bittencourt, 2008).

5. Participation of L-arginine/L-glutamine coupling in diabetes

From the above discussion, it seems clear that the development of diabetes is not simply a question of cytokine imbalance culminating in a redox disruption and consequent oxidative stress that disrupts or kills β -cells. This, in fact, raises another question: is beta cell susceptibility to stress solely a question of compromised antioxidant defence? If this were the case, it would appear preposterous that such a sophisticated cell remains 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.

In β -cells, pro-inflammatory cytokines induce the production of NO, synthesised from L-arginine, via a reaction catalysed by iNOS, whose functionality depends on NF- κ B-driven gene transcription and *de novo* enzyme synthesis. iNOS also utilises NADPH and O₂ as co-

substrates (**Fig. 1A**) and, physiologically, L-arginine is the limiting substrate for NO production. In addition to this, pancreatic β -cells express another L-arginine-metabolising enzyme, *i.e.* L-arginase (L-arginine amidinohydrolase, EC 3.5.3.1), which allows for the completion of urea production through the formation of L-ornithine and urea from L-arginine (Cunningham et al., 1997). Physiologic levels of L-arginase gene expression and activity have been measured in rat β -cells and the insulin-secreting cell line RINm5F (Cunningham et al., 1997; Malaisse et al., 1989; Cardozo et al., 2001; Rieneck et al., 2000). 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 (compare **Fig. 1A and 1B**). Consequently, L-arginase may impair NO production by limiting the availability of L-arginine for iNOS catalysis (Wu & Morris, 1998; Boucher et al., 1999; Mori & Gotoh, 2000). This notion is supported by the finding that inhibition of L-arginase results in enhanced NO synthesis in cytokine-activated cells (Chang et al., 1998; Tenu et al., 1999).

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 (Nussler et al., 1994; Hattori et al., 1994; Nagasaki et al., 1996), including β -cells (Flodstrom et al., 1995), *in vitro* as well as *in vivo*. L-Arginase activity may be increased in peritoneal macrophages after exposure to LPS (Currie, 1978), 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 functional (Granger et al., 1990). In clonal β -cells, IL-1 β increases L-arginase activity with concomitant increase in NO production (Cunningham et al., 1997), which suggests a kind 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 lymphocytes. While L-arginase activity may be induced by the “anti-inflammatory” Th2 cytokines IL-4, IL-6, IL-10, and IL-13 (Modolell et al., 1995; Waddington et al., 1998; Munder et al., 1999; Wei et al., 2000), 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-1 β and TNF α (Gill et al., 1996). 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 (Modolell et al., 1995; Corraliza et al., 1995), and by co-culturing murine macrophages with Th1 and Th2 T-cell clones (Munder et al., 1998). 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 (Modolell et al., 1995).

Because of the above statements, a Th1/Th2 lymphocyte dichotomy has been proposed to play a central role in the pathogenesis of type 1 diabetes (Rabinovitch & Suarez-Pinzon, 1998), whereas evidence suggests that the progression of the disease correlates with a Th1-type immune response (Currie, 1978; Granger et al., 1990; Simmons et al., 1996). Increased generation of NO following cytokine-elicited iNOS induction during insulinitis may contribute to β -cell destruction (Modolell et al., 1995; Morris et al., 1998). Therefore, competition between L-arginase and iNOS may be particularly important in protecting β -cells against the establishment of type 1 diabetes.

That macrophages exposed to LPS and IFN γ increase iNOS expression and NO production is well known. 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 (Boucher et al., 1994; Daghigh et al., 1994). Indeed, substantial amounts of this metabolite are released by LPS-treated rat alveolar macrophages (Hecker et al., 1995), 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 (SNOG), an adduct produced by the reaction of NO with GSH, are also inhibitors of L-arginase in many cell types (Daghigh et al., 1994; Knowles & Moncada, 1994), including β -cells (Cunningham et al., 1997). Hence, intermediates of NO synthesis, as well as NO itself, precisely coordinate a maximum of flux through iNOS in insulin-producing pancreatic cells (**Fig. 1**). Conversely, dexamethasone and dibutyryl cAMP block both iNOS and L-arginase expression, which is paralleled by a strong decrease of NO production (Gotoh & Mori, 1999). Additionally, macrophages treated with LPS and IFN γ undergo NO-dependent apoptosis, which may be prevented by L-arginase DNA plasmid transfection (Gotoh & Mori, 1999). In such cells, L-arginase I and II seem to play a role in determining the route(s) for NO-elicited outcomes.

Competition between L-arginase and iNOS has also been found in activated murine macrophages incubated with another L-arginase inhibitor, nor-L-NOHA (Tenu et al., 1999). 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 utilisation by L-arginase (Rutschman et al., 2001). Similar results have been obtained by using different cell types (Gotoh & Mori, 1999; Hecker et al., 1995). 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 (Wu & Morris, 1998; Stickings et al., 2002; Castillo et al., 1993), 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 insulinitis, since L-arginine concentrations are likely to be reduced at sites of inflammation due to the release of soluble L-arginase from infiltrating macrophages (Albina et al., 1990). Corroborating this proposition is the fact that IL-1 β -induction of NO synthesis in RINm5F insulin secreting 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 (Cunningham et al., 1997). 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-1 β that inhibits L-arginase expression in β -cells (Cardozo et al., 2001; Rieneck et al., 2000). In fact, a remarkable reduction in L-arginase expression has been recently observed during insulinitis in the NOD mouse model of type 1 diabetes (Rothe et al., 2002).

In the β -cell, NH_4^+ may contribute to L-arginine biosynthesis, through the concerted action of carbamoyl phosphate synthetase I, ornithine transcarbamoylase, argininosuccinate synthetase and argininosuccinate lyase that produce L-arginine (**Fig. 1B**). L-Glutamate is also believed to amplify glucose-induced insulin secretion in a K_{ATP} channel-independent way (Brennan et al., 2003). However, L-glutamate is, at the same time, an obligatory substrate for GSH synthesis, which, in turn, enhances the ATP/ADP ratio by optimising mitochondrial function and scavenges ROS/RNS leading to insulin secretion. L-alanine, may replenish the β -cell L-glutamate pool via an L-alanine aminotransferase-catalysed reaction. This explains why L-alanine is cytoprotective to β -cells against cytokine-induced apoptosis (Cunningham et al., 2005), *i.e.*, under cytokine-stimulated NO production,

L-alanine may provide L-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 L-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 utilise L-glutamine-derived L-glutamate to synthesise GSH, thus ensuring little L-glutamate can undergo oxidative deamination via glutamate dehydrogenase (GDH) in these conditions. 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 L-glutamine in the gut and release L-arginine into the blood (**Fig. 1 and 2**), although other tissues strongly express argininosuccinate synthetase and lyase but without any net delivery to the circulation (Vermeulen et al., 2007). In fasted humans, the contribution of L-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 (Vermeulen et al., 2007). A minor part of circulating L-arginine may also be provided by the enterocyte metabolism of proline, as stated in the Introduction. Consequently, if, by any chance, the flux through the coupled L-glutamine/L-arginine pathway between intestine and kidney is reduced or lost, then the knock on consequences for NO synthesis are severe (**Fig. 1**). L-Glutamate, however, is a unique source of GSH in β -cells, so that a disruption or hypofunctionality of intestinal-renal L-glutamine/L-arginine axis, would promptly decrease GSH synthesis thus reducing insulin release, leading to oxidative stress and β -cell death. On the other hand, L-glutamine which is a major and immediate L-glutamate precursor, is also a primary nutrient for the maintenance of immune cell function (Curi et al., 1999; Newsholme et al., 2003; Pithon-Curi et al., 2004). Hence, we believe that an immune response triggered by an immune or chemical challenge in a redox-sensitive subject (in which the expression/activity of antioxidant and GSH enzymes is low) might decrease the availability of L-glutamine for GSH generation in β -cells, leading to oxidative stress (**Fig. 1B**). Analogously, it seems likely that other situations, in which the circulating L-glutamine pool is severely endangered (Curi et al., 1999; Newsholme et al., 1987; Lagranha et al., 2008), such as in undernourishment, strenuous-exercise or cancer cachexia-associated muscle loss, chronic inflammatory diseases (including obesity), severe metabolic acidosis, major burns, polytrauma and bacteremia, should result in β -cell dysfunction.

L-Glutamine deficiency can occur during periods of critical illness. In patients with catabolic diseases, plasma and muscle L-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 L-glutamine concentration is lower than 50% of that in normal controls and it remains low for at least 21 days after the injury (Parry-Billings et al., 1990). Conversely, in LPS-endotoxemic rats, a single dose of L-glutamine, which is known to induce anti-inflammation via HSP70 expression (Wischmeyer et al., 2003; Singleton, K.D. & Wischmeyer, P.E., 2008; Hamiel et al., 2009; Zhang et al., 2009) has been shown to attenuate the release of TNF α and IL-1 β and to be associated with a significant

decrease in mortality due to the attenuation of pro-inflammatory type 1 cytokines (Wischmeyer et al., 2001), whereas L-arginine-enriched diet limits plasma and muscle L-glutamine depletion in head-injured rats (Moinard et al., 2006). Remarkably, however, **predominately Th1** (but not Th2) cell responses require the presence of optimal concentrations of L-glutamine (Chang et al., 1999). Since β -cell death that accompanies the onset of type 1 diabetes is an essentially Th1-elicited cytotoxic challenge, it is not unreasonable to suppose that the specific recruitment of Th1 cells may greatly enhance L-glutamine and L-arginine utilisation leading to an L-arginine deficit, which causes a reduction of insulin release and redox imbalance.

The positive actions of L-arginine on viability, antioxidant status and insulin secretion are likely to reflect, in large part, the importance of GSH and the glutathione disulphide (GSSG) reductase systems as the main lines of antioxidant defence in β -cells which are characterised by low levels of CAT and GSPx. In order to adequately provide GSH, β -cells may either regenerate GSH from GSSG via a GSSG reductase-catalysed reaction or synthesise it, *de novo*, through the concerted action of γ -glutamylcysteine synthetase (γ -GCS) and GSH synthetase, which are ATP-consuming enzymes (see **Fig. 2** for metabolic schemes). Regeneration of GSH from GSSG, which utilises NADPH as a co-factor but does not require ATP, is metabolically less expensive than the *de novo* synthesis from the constituent amino acids (L-glutamate, L-cysteine and L-glycine). However, unlike the majority of cell types, pentose phosphate shunt activity is relatively low in β -cells (Dröge, 2002), which is exacerbated by the high flux of glucose directed towards ATP production (Spinass, 1999). Therefore, β -cell NADPH must be obtained from the cytosolic malic enzyme (**Fig. 2B**), capable of converting malate to pyruvate with the concomitant production of NADPH from NADP⁺ (MacDonald, 1995). *De novo* GSH synthesis, on the other hand, is completely dependent on the supply of L-glutamate, not only because this amino acid is a constituent of the GSH molecule, but also because L-glutamate acts as an amino acid donor in the synthesis of serine, which can subsequently, be converted to L-glycine, via a reaction requiring tetrahydrofolate.

We have found that L-arginine significantly increased glucose consumption in β -cells, while decreasing lactate formation, regardless the presence or not of pro-inflammatory cytokines, (unpublished results, also see **Fig. 2B**). This may suggest that L-arginine is able to divert glucose from mitochondrial CO₂ production towards the formation of NADPH via the cytosolic malic enzyme so requiring that glucose-derived malate is transported from the mitochondrial matrix to the cytosol. Indeed, we believe that, in the presence of L-arginine, L-glutamate can be generated from both L-arginine and glucose (via 2-oxoglutarate formation and transamination) and is subsequently utilised for GSH synthesis (please, compare **Fig. 2B and 2C**). L-Arginine addition enhances the conversion of AMPK into its active phosphorylated form, thus favoring fatty acid oxidation and ATP synthesis while glucose metabolism is supporting malate formation and L-glutamate formation for NADPH and GSH generation respectively. This requirement, however, results in a reduction in stimulus-secretion coupling and the associated insulin release.

We have also observed that NOS-2 expression is stimulated by the cytokine cocktail (which enhances iNOS activity) but NO synthesis was not enhanced by changing L-arginine in the culture medium. This suggests that iNOS is saturated with L-arginine which, in turn, results in elevated urea production. This shunt in L-arginine metabolism efficiently preserves β -cell redox status by favoring the production of GSH in conditions which generate excessive levels of NO (**Fig. 2C and 2D**).

Fig. 2. 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-cantered machinery to produce NO, which potentiates insulin secretion physiologically. At the same time, insulin-secreting pancreatic cells utilise 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- κ B-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 I (CPS), ornithine transcarbamoylase (OTC), argininosuccinate synthetase (ASS) and argininosuccinate lyase (ASL), which, dramatically enhances the flux of L-glutamate towards NO production. In the presence of an inflammatory NF- κ B-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 L-glutamate, L-cysteine and L-glycine, once 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 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 (glutamate-cysteine ligase) and GSH synthetase in the GSH biosynthetic pathway. Therefore, muscle-derived L-alanine and L-glutamine constitute the principal sources of L-glutamate for GSH synthesis. Because of this, any reduction in L-arginine supply to β -cells accounts for a rapid shift in L-glutamate metabolism from GSH synthesis towards NO production. For instance, during Th1-elicited immune responses, the concerted enhancement of NF- κ B-mediated (*) expression of ASS, ASL and iNOS dramatically boosts NO production from L-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 interleukin-6 (IL-6) may alleviate NO toxicity by enhancing L-arginase expression that diverts L-arginine to the formation of L-ornithine and urea. At the same time, intracellular expression of the 70-kDa family of heat shock proteins (HSP70), which blocks a surplus activation of NF- κ B-dependent genes, is cytoprotective because it warrants an equilibrium for NO production via NF- κ B-dependent iNOS expression thus avoiding NO cytotoxic effects. 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 L-glutamine metabolism, towards the production of L-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 utilisation 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. Since physical exercise stimulates L-glutamine flux towards L-arginine production, peaks IL-6 secretion by the stretching skeletal muscle and induces HSP70 expression throughout the body tissues, exercise continues to be the cheapest and most efficient way of preventing type-1 diabetes onset. Arrow widths indicate the intensity of the metabolic flux through each pathway.

L-Arginase is normally associated with a K_m value for L-arginine that is much higher than that of iNOS but a greater V_{max} value compared with iNOS (Mori, 2007), so that the V_{max}/K_m ratios of both enzymes are close to each other and thus these enzymes may be expected to compete for L-arginine equally in β -cells. In our hands, iNOS seemed to be saturated in β -cells, regardless of the presence of inflammatory cytokines, so that β -cell urea production is able to furnish L-ornithine and thus L-glutamate for GSH synthesis in appropriate conditions. Moreover, L-arginine may protect β -cells via the induction of haem oxygenase (HO-1) expression (data not shown). HO activity is an important detoxifying enzyme, due to its ability to scavenge haem groups thus providing redox protection (Abraham & Kappas, 2008). 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 recently been reported to induce insulin secretion and to improve *in vivo* function of β -cells after transplant (Abraham & Kappas, 2008). Moreover, the long-lasting expression of this enzyme has been shown to delay the progression of type 1 diabetes in NOD mice (Li et al., 2007). Hence, L-arginine can be recognised as an antioxidant in its own right, being comparable with known antioxidant stimuli, such as phytochemical supplements (Velmurugan et al., 2009).

Furthermore, and interestingly, chronic hyperlactataemia, in which high plasma levels of lactate block intestinal proline oxidase activity leading to severe hypocitrullinaemia and hypoargininaemia (Dillon et al., 1999), has been described as an independent risk factor for diabetes development, with lactate being an important factor for maintaining insulin resistance (DiGirolamo et al., 1992; Lovejoy et al., 1992). Conversely, L-arginine supplementation to critical care patients did induce L-glutamine rise in the plasma (Loi et al., 2009), which may be related to the fact the L-arginine supplementation spares plasma glutamine pools.

In synthesis, L-arginine derived from the kidney (**Fig. 1**) is the physiological substrate for the NF- κ B-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 synthesise GSH from L-glutamate, L-cysteine and L-glycine, because regeneration of GSH from 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-derived 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 L-alanine and L-glutamine constitute the principal sources of L-glutamate for GSH synthesis in order to spare β -cell L-arginine stores. In fact, previous reports from our laboratory have highlighted the importance of L-glutamine and L-alanine for GSH generation, insulin secretion and protection against pro-

inflammatory cytokines (Brennan et al., 2003; Brennan et al., 2002; Cunningham et al., 2005). Because of this, **any reduction** in L-arginine supply to β -cells accounts for a rapid shift in L-glutamate metabolism from GSH synthesis towards NO production. For instance, during Th1-elicited immune responses (e.g. as in Fig. 2C and 2D), the concerted enhancement of nuclear factor NF- κ B-mediated expression of ASS, ASL and iNOS dramatically boosts NO production from L-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 (T2-CK) may alleviate NO toxicity by enhancing L-arginase expression that deviates L-arginine to the formation of L-ornithine and urea.

6. Psychological stress and the role peripheral sympathetic nervous system-histamine-CRH axis activation in type 1 diabetes

It has long been recognised that stressful situations are closely related to the onset of type 1 diabetes. In fact, many stressful conditions that are associated with immune system imbalances, including psychological ones, are associated with the incidence of type 1 diabetes (Soltesz, 2003; Dahlquist, 2006). Indeed, it has recently been shown that stressful life events and psychological dysfunctions dramatically augment the likelihood of the incidence of type 1 diabetes in children and adolescents (Sipetic et al., 2007). 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 (Elenkov et al., 1999). CRH stimulates lymphocyte proliferation (McGillis et al., 1989; Jessop et al., 1997) and secretion of IL-1 β and IL-2 by mononuclear cells isolated from the peripheral blood of healthy subjects (Singh & Leu, 1990). Peripheral CRH exerts a pro-inflammatory effect in autoimmune diseases with a selective increase in Th1-type responses, which is mediated by an NF- κ B-dependent pathway (Benou et al., 2005). 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 (Elenkov et al., 1999). In fact, diabetes is associated with increased basal hypothalamus-pituitary-adrenal (HPA) activity and impaired stress responsiveness (Chan et al., 2005). Therefore, psychological stress may selectively activate Th1 lymphocytes that mediate type-1 cytokine-induced iNOS expression, exacerbated NO production and β -cell cytotoxicity. Enhanced Th1 activity, in turn, increases L-glutamine utilisation with the consequent shift of L-glutamate metabolism from GSH biosynthesis towards NO production, as discussed above (Fig. 2 and 3).

Taken together, these findings suggest that psychological stress may have a dual and cross-potentiating role in determining the onset of type 1 diabetes: an immunoinflammatory (Fig. 3) and a metabolic one (Fig. 2C and 2D). 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 (Wu et al., 2004), without increasing urea

Fig. 3. Psychological stress and 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. Peripheral sympathetic nerve-derived CRH released under acute psychological stressful situations is capable of stimulating mast cells and Th1 lymphocytes, which arm an immunoinflammatory response. Auto-reactive Th1 cell subset and its cytokine products (type 1 cytokines, T1-CK) raised against islet β -cell antigen(s) mediate the activation of macrophages and Th1 lymphocytes, favouring insulinitis. Additionally, other predisposing factors may also exacerbate β -cell injury and the onset of type 1 diabetes mellitus (T1DM).

levels (Wilmore, 2004). Curiously, intraperitoneal L-arginine injection, where the physiological coupling of L-glutamine/L-arginine through the intestinal-renal axis is bypassed, does **not** improve diabetes in animal models. On the contrary, it seems to worsen it (Mohan & Das, 1998), while **oral** administration of L-arginine to alloxan-treated rats restores blood glucose and insulin levels (Vasilijevic et al., 2007). Oral L-arginine administration has also been shown to improve, but not completely, peripheral and hepatic insulin sensitivity in type 2 diabetes (Piatti et al., 2001), where oxidative stress (Carvalho-Filho et al., 2005; Oliveira et al., 2003; Hirabara et al., 2006) and NO overproduction (Newsholme et al., 2007; Carvalho-Filho et al., 2005) are also involved. If this is so, nutritional management of L-glutamine and/or L-arginine, **enterally** administered in order to allow for the physiological re-establishment of L-glutamine/L-arginine homeostasis (Vermeulen et al., 2007), may rescue β -cell redox balance in ongoing type 1 diabetes. Additionally, skeletal muscle is a major site for L-glutamine synthesis in the human body and contains over 90% of the whole-body L-glutamine pool. Quantitative studies in humans

(Newsholme et al., 2003) have demonstrated that, in the postabsorptive state, 60% of the amino acids released comprise L-alanine plus L-glutamine (**Fig. 1A**). Therefore, moderate physical exercise, which is known to accelerate the rate of L-glutamine delivery into the circulation, may be of value in protecting L-glutamine/L-arginine metabolic coupling between the gut and β -cells.

7. Influence of regular physical exercise in L-arginine/L-glutamine coupling in β -cells

During physical exercise sessions, pro-inflammatory cytokine production is downregulated and anti-inflammatory cytokines, such as IL-1 receptor antagonist (IL-1ra), IL-10 and IL-6, are upregulated (Drenth et al., 1995; Nieman & Pedersen, 1999; Rohde et al., 1997). In this sense, IL-6 seems to play a capital 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 (Pedersen & Hoffman-Goetz, 2000; Febbraio et al., 2002; Pedersen & Steensberg, 2002; Pedersen et al., 2001). Most studies have also 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 (Pedersen et al., 1998; Suzuki et al., 2000). 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-induced IL-6 release downregulates 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- κ B activation, we believe that moderate exercise-induced IL-6 production may suppress NF- κ B-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 L-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 type 1 diabetes.

Even though the effects of IL-6 on β -cells remains a matter of debate and controversies (Wadt et al., 1998), it has been found that IL-6 hinders the development of type 1 diabetes in different mouse models (Campbell et al., 1994; DiCosmo et al., 1994). Moreover, IL-6 has proven 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 (Choi et al., 2004). Altogether, the above propositions support an important protective effect of exercise-dependent muscle-derived IL-6 on β -cells against the development of diabetes. Moreover, exercise-induced HSP70 expression in non-muscular cells may have a critical influence in maintaining an anti-inflammatory status, as discussed above. However, exercise-induced HSP70 in pancreatic β -cells has never been addressed. Therefore, we

are currently evaluating the effects of acute and chronic (training) exercise sessions (swimming) on HSP70 pathways and L-glutamine/L-arginine coupling enzymes in animal pancreatic islets and isolated β -cells.

8. Conclusion

Continued supply of L-arginine, physiologically provided by the metabolism of L-glutamine via the intestinal-renal axis and from skeletal muscle, which is enhanced during exercise, is essential for β -cell functional integrity and, indeed, for β -cell defence. The dysregulation of immune system function, characteristic of Th1-elicited β -cell toxicity and impaired insulin secretion, which accompany the onset of type 1 diabetes, may be triggered when an individual faces a strong **psychological stress** that determines an enhanced L-glutamine utilisation by Th1 lymphocytes. The oxidative stress that takes place upon reduced intracellular GSH levels allows for the activation of NF- κ B, 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 type 1 diabetes. Physical exercise, on the other hand, is capable of inducing a huge production and release of IL-6, which is a key anti-inflammatory mediator that suppresses NF- κ B-dependent responses. Moreover, exercise-elicited activation of HSP70 biochemical pathways completely blocks NF- κ B 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 L-glutamine supply thus normalizing pancreatic production of NO from kidney-derived L-arginine, and not from L-glutamate which is necessary for GSH synthesis and antioxidant defence. Thus 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. As previously argued (Krause & Homem de Bittencourt, 2008), we advocate that present-day levels of physical activity and dietary patterns (Simopoulos, 2006; Wisloff et al., 2005) 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 (favouring energy conservation and storage). As a corollary, unless humans enhance their pattern of physical activity, diabetes will become more and more of a risk factor in the population. Therefore, the notion that β -cells are solely bystanders of oxidative stress-mediated cell toxicity because their antioxidant defences fail in managing physiological stress is an unfortunate misconception. Since the L-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 dysfunction or death in diabetes. Additionally, although the possibility of pharmacologically exploiting Th1/Th2 duality relative to L-arginine metabolism may open new avenues for diabetes therapeutics, physical exercise is still the cheapest and easiest physiological measure to avoid the onset and/or worsening of diabetes. In summary, if the prevention of diabetes is dependent on HSP70 expression and both restoration of adequate L-arginine supply to β -cells and blockage of NF- κ B overstimulation, moderate physical exercise is presented as the most convenient solution for these two lacunes.

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This book is intended as an overview of recent progress in type 1 diabetes research worldwide, with a focus on different research areas relevant to this disease. These include: diabetes mellitus and complications, psychological aspects of diabetes, perspectives of diabetes pathogenesis, identification and monitoring of diabetes mellitus, and alternative treatments for diabetes. In preparing this book, leading investigators from several countries in these five different categories were invited to contribute a chapter to this book. We have striven for a coherent presentation of concepts based on experiments and observation from the authors own research and from existing published reports. Therefore, the materials presented in this book are expected to be up to date in each research area. While there is no doubt that this book may have omitted some important findings in diabetes field, we hope the information included in this book will be useful for both basic science and clinical investigators. We also hope that diabetes patients and their family will benefit from reading the chapters in this book.

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