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Review

The harmony of the spheres: inducible nitric oxide synthase and related genes in pancreatic beta cells

D. L. Eizirik[1,2], M. Flodström[2], A. E. Karlsen[3], N. Welsh[2]

[1] Department of Metabolism and Endocrinology, Vrije Universiteit Brussel, Brussels, Belgium
[2] Department of Medical Cell Biology, Uppsala University, Uppsala, Sweden
[3] Steno Diabetes Center, Gentofte, Denmark

Summary The radical nitric oxide (NO) is a possible mediator of pancreatic beta-cell damage in insulin-dependent diabetes mellitus (IDDM). NO is produced by the enzyme nitric oxide synthase (NOS), in a reaction where arginine is the main substrate. There are different isoforms of NOS, but in the context of immune mediated beta-cell damage the inducible form of NOS (iNOS) is the most relevant. The beta-cell iNOS is similar and encoded by the same gene on chromosome 17 as the iNOS expressed in macrophages and other nucleated cells. iNOS activation depends on gene transcription and de novo enzyme synthesis, and NO seems to induce a negative feedback on iNOS expression. While iNOS mRNA is induced by interleukin-1β (IL-1β) alone in rodent insulin-producing cells, a combination of two (IL-1β + interferon γ) (IFN-γ) or three (IL-1β + IFNγ + tumour necrosis factor α) cytokines is required for iNOS activation in human pancreatic islets. The promoter region of the murine iNOS gene has at least 25 binding sites for different transcription factors, and the nuclear transcription factor $\varkappa$B is necessary for cytokine-induced iNOS transcription in both rodent and human pancreatic islets. The nature of other transcription factors relevant for iNOS regulation in these cells remains to be determined. Induction of iNOS is paralleled by induction of several other cytokine-dependent genes in beta cells, including argininosuccinate synthetase, cyclooxygenase and manganese superoxide dismutase. Some of these genes may contribute to beta-cell damage, while others are probably involved in beta-cell defence and/or repair. Regulation of iNOS and other related genes in beta cells is complex, and differs in several aspects from that observed in macrophages. There are also important differences in iNOS regulation between rodent and human pancreatic islets. A detailed knowledge of the molecular regulation of these genes in beta cells may be instrumental in the development of new approaches to prevent beta-cell destruction in early IDDM. [Diabetologia (1996) 39: 875–890]

Keywords Nitric oxide, nitric oxide synthase, promoter, transcription factor, nuclear factor $\varkappa$B, pancreatic islets, beta cells, insulin-producing cells, insulin-dependent diabetes mellitus, superoxide dismutase.

Corresponding author: Dr. D. L. Eizirik, Deparment of Metabolism and Endocrinology, Vrije Universiteit Brussel, Laarbeeklan 103, B-1090 Brussels, Belgium

Abbreviations: iNOS, Inducible nitric oxide synthase; NO, nitric oxide; IDDM, insulin-dependent diabetes mellitus; IL-1β, interleukin-1β; IFNγ, interferon γ; TNFα, tumour necrosis factor α; NF-$\varkappa$B, nuclear transcription factor $\varkappa$B; I$\varkappa$B, inhibitor of NF-$\varkappa$B; γ-IRE, interferon γ response element; NOD, non-obese diabetic; BB, bio-breeding; ISRE, IFNα-stimulated response element; HRE, hypoxia-responsive element; IRF, interferon regulatory factor; EMSA, electrophoretic mobility shift assay; PDTC, pyrrolidine dithiocarbamate; LPS, lipopolysaccharides; PKC, protein kinase C; SAPK/JNK, stress activated/c-jun NH_2-terminal protein kinases; MAPK, mitogen-activated protein kinase; PAK, p21 (Cdc 42/Rac) activated kinase; MEK, MAPKkinase; CAPK, ceramide-activated protein kinase; JAK, *Janus* kinases; ATF/CRE, activating transcription factor/cyclic AMP responsive element; DAG, diacylglycerol; PMA, phorbol 12-myristate 13-acetate; AS, argininosuccinate synthetase; iCOX, inducible form of cyclooxygenase; MnSOD, manganese superoxide dismutase; hsp, heat shock protein; IL-1R, interleukin-1 receptor; STAT, signal transducer and activator of transcription; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; TGF, transforming growth factor; SSRE, shear stress response element; TLCK, tosyl-L-lysine chloromethylketone; GAPDH, glyceraldehyde-3-phosphate dehydro genase.

Nitric oxide (NO) is a small, short-lived and highly reactive radical, which mediates diverse biological functions, such as vasodilation, neurotransmission, antimicrobial and antitumoural activities [1, 2]. NO is produced by the enzyme nitric oxide synthase (NOS), in a reaction where arginine and oxygen are converted into citrulline and NO. There are two major classes of the enzyme [3]: a) the constitutive isoforms, present mostly in neurons and endothelial cells (types 1 and 3); b) the inducible isoform (iNOS; type 2), originally described in macrophages, but now shown to be expressed in a large variety of mammalian cells [4].

iNOS requires *de novo* synthesis following cellular stimulation by cytokines or bacterial lipopolysaccharides (LPS) and the enzyme produces larger amounts of NO than the constitutive isoforms. NO exerts lethal effects on several pathogens, including protozoans, fungi, bacteria and viruses [4]. It has been proposed that this radical is part of an "innate" defence system against invading microorganisms [5]. This system is activated at the cellular level, even before the immune system is geared for response. If this is the case, it may explain why so many nucleate cells express iNOS upon proper cytokine or LPS stimulus. It is noteworthy that there are important species and tissue differences in the requirement for stimulus leading to iNOS induction. Moreover, while human hepatocytes [6] and pancreatic islets [7, 8] readily produce large amounts of NO in response to cytokines, human macrophage iNOS is restricted in its expression and seems to produce less NO than rodent macrophages [9].

Autoimmune diseases are characterized by a failure of self-tolerance, leading the immune system to attack and destroy endogenous tissues. In insulin-dependent diabetes mellitus (IDDM), hyperglycaemia is probably the outcome of a long-term negative balance between immune-mediated beta-cell damage [10] and beta-cell repair/regeneration [11]. Cytokines may be part of the armament used by the immune system to destroy beta cells in IDDM [12, 13]. Under in vitro conditions interleukin-1 (IL-1), alone or in combination with tumour necrosis factor-α (TNFα) or interferon-γ (IFNγ), induces functional suppression and damage to rodent pancreatic islets [12–14]. Human islets are less sensitive to the deleterious effects of cytokines, but exposure of these islets for several days to IL-1β + TNFα + IFNγ also induces beta-cell functional impairment [7, 8] and islet-cell loss [15, 16].

In the case of rodent islets, NO generation seems to be the main mechanism behind cytokine-induced beta-cell dysfunction. Southern and co-workers [17] showed that iNOS blockers protect rat islets against inhibition by cytokines, and these findings have been confirmed by several other groups (reviewed in [18]). Two of the most important effects of NO in rodent islets are inhibition of the mitochondrial enzyme aconitase, leading to decreased oxidative metabolism and ATP production [19, 20], and induction of nuclear DNA damage [21, 22]. Based on these data, it has been suggested that NO is an important mediator of beta-cell destruction in IDDM [23, 24]. Human islets are more resistant than rodent islets to the suppressive effects of cytokine- and chemically-generated NO [8, 25]. However, recent data suggest that higher concentrations of the radical induce damage to human islet cell DNA and may lead to cell death [26].

IL-1β alone induces transcription of iNOS mRNA both in insulinoma cell lines [27] and in adult rat islets [18, 28], while three cytokines (IL-1β + TNFα + IFNγ) are required to induce expression of this mRNA in human islets [8]. A major issue that remains to be clarified is the molecular regulation of iNOS mRNA expression in pancreatic beta cells. In recent years there have been important advances in the field, and this will be the main focus of the present review. Whenever possible, observations obtained in rodent islets and insulin-producing cells will be compared with data obtained in human islets.

iNOS gene structure

Cloned and expressed NOS cDNAs. Three different forms of NOS genes have been cloned and expressed. The NOS1 gene encodes one of the two constitutively expressed forms cloned from neuroectodermal cells (nNOS). It is a 161-kDa protein, translated from a 10 kilobase (kb) mRNA [29, 30]. The other constitutively expressed NOS, cloned from endothelial cells (eNOS), is transcribed as an approximately 4.7-kb mRNA encoded by the NOS3 gene, and translated into a 133-kDa protein [31–33]. nNOS has been associated with neuronal signal transduction [34] and eNOS with regulation of vascular tone [1, 35], respectively. They share approximately 60 % amino acid sequence similarity [31]. The inducible form (iNOS) was originally cloned from IFNγ and LPS stimulated mouse macrophages [36–38], and shows only 50 % amino acid sequence similarity/identity to the constitutive NOS isoforms. It is encoded by the NOS2 gene, and represented as an approximately 4.4-kb mRNA that translates into a 131-kDa protein [39, 40, 41]. The three iNOS forms show homology with mammalian cytochrome P450 reductase in the C-terminal half (30 %), where the co-factor binding-sites are located [29]. Figure 1 shows a schematic alignment of the three NOS genes/mRNAs. Since the original cloning from mouse macrophages [36–38], iNOS expression has been demonstrated in several other rat and human tissues, including hepatocytes, chondrocytes, endothelial cells, smooth myocytes, megakaryocytes, thyrocytes, mesangial cells

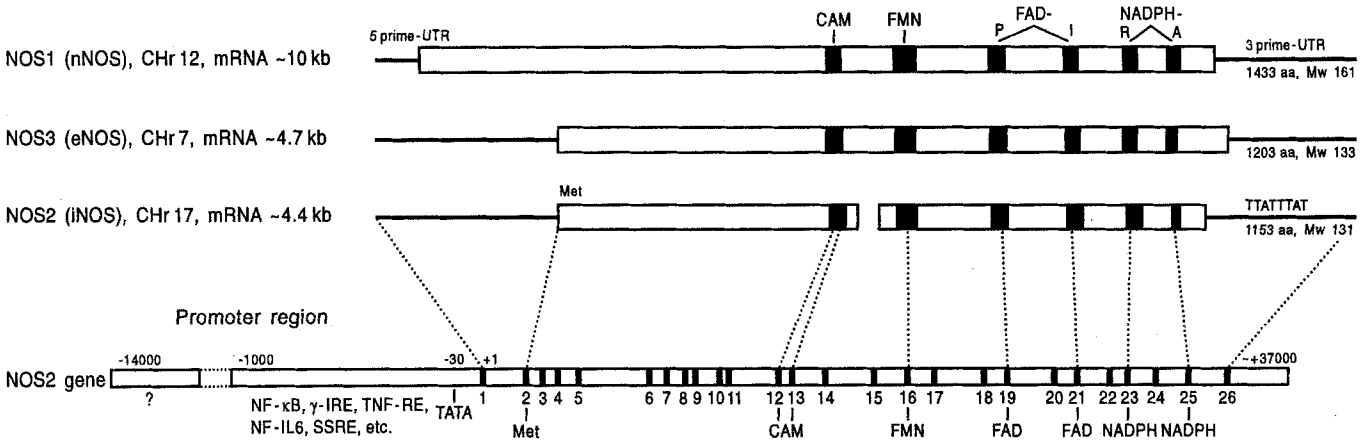

Fig. 1. Schematic representation of the three human isoforms of NOS (for references see text). Upper panel: comparison of the three NOS mRNAs, showing the coding regions for the different co-factor binding sites, including CAM (calmodulin), FMN (flavin mononucleotide), FAD (flavin adenine dinucleotide-pyrophosphate (P) and -isoalloxazine (I)) and NADPH (reduced nicotinamide adenine nucleotide phosphate-ribose (R) and -adenine (A)). Lower panel: schematic representation of the human iNOS gene [41] showing the position of the 26 exons (■) and the promotor region with some of the hitherto identified consensus sequences for binding of the transcription factors NF-$\varkappa$B (nuclear factor-$\varkappa$B), γ-IRE (interferon-γ-responsive element), TNF-RE (tumour necrosis factor responsive element), NF-IL6 (nuclear factor interleukin 6) and a SSRE (shear-stress responsive element). These, and several additional transcription elements (described in the text), have also been demonstrated within 1 kb from the TATA box in the mouse iNOS gene. However, while significant promoter activity in mice is located in this 1 kb area, the promoter activity of the human iNOS gene seems to extend up to 16 kb upstream of the TATA box (as described in the text). The TATA box precedes the mRNA initiation site (exon 1) by 30 nucleotides. The primary translation startsite (Met) and corresponding AUG site (exon 2) is shown, but other startsites may also exist (see text). UTR, Untranslated region of mRNA

and beta cells (reviewed in [42]). In their active form, the three NOS are homodimers, containing flavine adenine dinucleotide (FAD) and flavine mononucleotide (FMN) and requiring tetrahydrobiopterin and reduced nicotinamide adenine nucleotide phosphate (NADPH) for their activity [43]. This requirement for co-factors explains the high homology in the 3′-half of the coding region among the different NOS forms. Both constitutive NOS forms are calcium and calmodulin dependent for their enzymatic activity, which is regulated by calcium influxes promoting the binding of calmodulin to the pre-existing NOS. In contrast, calmodulin is bound to the iNOS with such high affinity that calcium fluxes have little effect on the enzymatic activity. The iNOS, producing high amounts of NO for a prolonged time period following de novo synthesis, is thus mostly regulated at the transcription/translation level by a variety of inflammatory stimuli [44]. Two-dimensional gel analysis of recombinant rat islet iNOS expressed in a human fibroblast 293-cell line, also suggests the possibility of post-translational control by phosphorylation [28].

As described above, iNOS has been associated with cytokine-mediated beta-cell dysfunction and destruction. Based on the findings that iNOS expression in some cells, e.g. murine macrophages, is induced by LPS and IFNγ [36–38], while iNOS expression in other cells, e.g. beta-cells, requires IL-1β (rat islets) or a combination of IL-1β + IFNγ or IL-1β + IFNγ + TNFα (human islets) to be induced [7, 8], it was speculated that different isoforms of iNOS, with different and potential beta-cell specific tissue-expression patterns, might exist. If this was the case, a beta-cell specific iNOS inhibition would represent a potential strategy for preventing IDDM. Cloning of iNOS from rat islets and rodent beta-cell lines [28], as well as human islets (Karlsen et al., unpublished data), however, revealed that the iNOS induced by cytokines in the islets is similar and encoded by the same gene [45] as the iNOS expressed in macrophages and other tissues [4, 42].

Chromosomal localization. The chromosomal position of the three different NOS genes have been located. The nNOS (NOS1) maps to position q24.2–24.31 on human chromosome 12 [46], cNOS (NOS3) to position q35–36 on chromosome 7 [47] and iNOS to position p11.2–q12 on human chromosome 17 [48]. The latter region is synthenic to the region on mouse chromosome 11, where the iNOS gene has been mapped [45]. Mouse chromosome 11 has previously been associated with IDDM in the spontaneously diabetic non-obese diabetic (NOD) mouse, through a polymorphic region (idd4) [49]. Interestingly, the iNOS gene is located in the middle of this idd4 region [45]. Furthermore, iNOS mRNA and NO have been detected in islets from both NOD mice [50] and bio-breeding (BB) rats [51] around the time of onset of spontaneous diabetes. Thus, a polymorphism in the NOS2 gene would represent an attractive candidate for idd4. The existence of strain

differences with regard to IL-1β-induced iNOS expression, NO production and beta-cell function has been recently demonstrated among different rat strains, but there were no polymorphisms in the rat iNOS gene, as evaluated with the use of 20 different restriction enzymes [52]. Minor differences in the cloned human iNOS cDNA sequences, potentially representing polymorphisms in the gene, have been found. However, association with diabetes prevalence has hitherto not been reported.

Most of the available cloning and chromosomal mapping data suggests the presence of only one iNOS gene within the genome. However, the presence of at least three iNOS-related genes in the murine and human genome has been proposed [53–56]. Thus, two new genomic sequences encoding iNOS-related genes have been mapped to human chromosome 17 between bands 13.1 and q25, including the region encoding NOS2 [55]. In a recent paper Xu and colleagues [56] cloned and sequenced a partial region (exon 22 region) of three of the human chromosome 17 encoded NOS2-like sequences, and in addition mapped another NOS2-like sequence to human chromosome 14. It remains to be determined whether any of these multiple NOS2-like sequences present in the human genome are pseudogenes or authentic, transcriptionally active genes, capable of translation into novel NOS proteins. It is noteworthy that the cosmids encoding these NOS2-related genes only contain the 3′ end of the coding region corresponding to the NOS2 cDNA sequence encoding the cofactor binding consensus sites. It is thus suggested that they may represent partially duplicated sequences [56]. Nevertheless, a differential expression and induction of two different iNOS mRNAs has been shown in the rat kidney genome [54]. The potential presence of genomic sequences, including promoter regions and coding regions for additional NOS2 related isoforms, some in close proximity to the NOS2 gene, may contribute to the complex machinery controlling NO synthesis.

5′ Region and promoter elements in the iNOS gene. Genomic cloning of the human iNOS gene revealed a gene of approximately 37 kb in length, consisting of 26 exons and 25 introns. The transcription initiation site was mapped 30 bp downstream of a TATA sequence and the translation start site is located in exon 2 [41] (Fig. 1). Considering that several different agents, such as cytokines, cyclic AMP, LPS, glucocorticoids, prostaglandin E2, picolinic acid etc., up- or down-regulate iNOS expression in different tissues, it is not surprising that multiple positive and negative regulatory elements, responsive to numerous transcription factors, have been identified in the mouse and human iNOS promoter region. In the mouse iNOS gene these include transcription factor motifs for IFNγ-response element (γ-IRE) (ten copies), γ-activated site (GAS) (three copies), nuclear factor-$\varkappa$B (NF-$\varkappa$B) (two copies), IFNα-stimulated response element (ISRE) (two copies), activator protein 1 (AP1) (two copies), TNF response element (TNF-RE) (two copies), nuclear factor interleukin 6 (NF-IL-6) (one copy), shear stress response element (SSRE) (one copy), X box (one copy) and hypoxia-responsive enhancer (HRE) (one copy) [57–60]. Using a mouse genomic library, cosmid clones containing two positively regulating regions, –48 to –209 (region 1) and –913 to –1029 (region 2), upstream of a putative TATA box of the macrophage iNOS have been identified [58]. Region 1 contains LPS-related responsive elements, including a binding site for NF-IL-6 and the $\varkappa$B binding site for NF-$\varkappa$B. Region 2, which by itself (in contrast to region 1) does not promote transcription activity, but potentiates/augments the promoting activity of region 1, contains motifs of IFN-related transcription factors. This is in full concordance with the effect of LPS and IFNγ on iNOS induction in the RAW murine macrophage cell-line, especially taking into account negatively regulating DNA sequences (unpublished data) upstream of region 2 [58]. Similar data regarding the mouse promoter was also reported by another group [57]. This group, however, found an important difference in the induction pattern between the RAW murine cell line and that of primary mouse peritoneal macrophages. In contrast to the RAW cells, the primary macrophages do in fact respond to IFNγ alone by producing NO [61], whereas they do not respond to LPS alone [57]. Furthermore, while in macrophages a downstream NF-$\varkappa$B site extending from position –85 to –76 is necessary for LPS-induced iNOS expression [4, 57], in vascular smooth muscle cells an upstream $\varkappa$B site (position –890 to –1002) is of key importance for the combined effects of cytokines on iNOS expression [62]. Thus, even taking into account that the iNOS promoter region is identical among different cell types, it seems that diverse regulating regions are used by these cells following different stimuli. This suggests that the complex synergistic induction and suppression of iNOS cannot be safely extrapolated between distinct cell types, for instance from macrophages (the most studied cell type) to pancreatic beta cells or between transformed cell lines and primary cells. The same is probably true for results obtained in similar cells from different species, i. e. beta cells from humans or rodents (see below). Thus, to identify all the relevant promoter regulating regions in the human and rodent beta-cell iNOS gene it will be necessary to transfect these cells with different promoter-reporter constructs, as previously done for macrophages [57] and vascular smooth muscle cells [62].

Analysis of the human iNOS promoter revealed similar positions of the TATA box, transcription- and translation start-sites as in the murine iNOS

promoter [41, 59, 63]. There are more than 30 putative transcription factor motifs in the first 1500 bp upstream of the human iNOS transcription start site, including several copies of NF-$\varkappa$B, IFNγ responsive elements and TNF responsive elements, and this region has a 47% sequence homology to the mouse iNOS promoter [64]. However, the functional promoter activity of the 1.5-kb region in the human iNOS has not been demonstrated experimentally. In this context, it is surprising that the human iNOS promoter in a cytokine responsive human liver epithelial cell line failed to show promoter activity in response to a mixture of TNF, IL-1β and IFNγ, in promoter constructs extending up to 3.8 kb upstream of the transcriptional start site. However, there was a threefold and fivefold induced reporter activity with a 5.8-kb and 7.0-kb construct, respectively, while a 16-kb construct resulted in tenfold induction [64]. These results suggest that human iNOS is regulated by a uniquely complex promoter region, requiring cytokine-responsive elements upstream of 3.8 kb to increase transcriptional activity. This is in contrast to the murine iNOS promoter, where only 1 kb of the proximal 5′ flanking region is required to augment transcriptional activity [4].

Interestingly, a recent study demonstrated the presence, and up-regulation by cytokines, of smaller amounts of TATA-independent human iNOS mRNA transcripts from the same gene described above, as well as the presence of alternative splicing in the 5′ untranslated region of the mRNA [63].

3′ Region and mRNA stability. There is no structural diversity in the 3′-untranslated region of iNOS, as evaluated by RT-PCR [63], and both murine and human iNOS mRNA has a conserved region in the 3′ untranslated end with high homology to an octanucleotide sequence UUAUUUAU [40]. This sequence has previously been shown to decrease mRNA stability in the TNF gene, probably by allowing binding of labile cycloheximide-sensitive proteins (cycloheximide is an inhibitor of protein synthesis) [65, 66]. Increased intracellular calcium inhibits iNOS activity in IL-1-treated human articular chondrocytes by reducing iNOS mRNA stability [67]. Similarly, transforming growth factor β (TGFβ) decreases iNOS mRNA content in macrophages partly by increasing iNOS degradation [68]. On the other hand, cycloheximide prolongs iNOS mRNA half-life in smooth muscle cells and chondrocytes by preventing the synthesis of proteins involved in iNOS mRNA degradation [66]. iNOS mRNA has a short half-life in insulin-producing cells, and when IL-1β-induced iNOS transcription in beta cells is arrested by actinomycin D (a blocker of gene transcription), there is a 50% decrease in cellular iNOS mRNA content after 2 h [69]. As a whole, these findings suggest a role for mRNA stability in the regulation of cellular iNOS mRNA content, and may be one of several factors responsible for the differences in iNOS mRNA kinetics reported among different tissues and species.

Regulation of iNOS mRNA expression in pancreatic islets

When rat islets are exposed to IL-1β there is detectable iNOS mRNA expression after 4 h [18]. Similarly, when insulin-producing HIT [70] or RIN cells [69] are treated with IL-1β or TNFα + IFNγ, nitrite production and iNOS expression increases after 4 h and peaks after 6 h. Subsequently (after 12–48 h) there is a progressive decrease in iNOS expression and nitrite production, in spite of the continuous presence of IL-1β [69]. This is probably due to a negative feedback by NO on iNOS transcription, as suggested by the observation that NO donors inhibit cytokine-induced iNOS expression in insulin-producing cells [69], glial cells [71, 72] and RAW 264.7 cells [73]. NO decreases iNOS expression by preventing NF-$\varkappa$B activation [72, 74], an effect mediated by induction and stabilization of I-$\varkappa$B [74] (see below for discussion on role of NF-$\varkappa$B for iNOS transcription in beta cells). In macrophages NO can also inhibit iNOS enzyme activity by interacting with enzyme-bound haem [75]. Considering that excessive production of NO may lead to cell death, NO-induced negative feedback on its own production may be part of the cellular protective mechanisms against the deleterious effects of the radical.

The pancreatic islet contains a heterogeneous cell population, making it difficult to determine the cellular source(s) of NO production. There is now evidence suggesting that differentiated beta cells [76] and clonal insulin-producing hamster (HIT [21, 27, 70]) and rat (RINm5F [23, 69, 77]) cells can be stimulated to produce large amounts of NO. That iNOS expression is indeed induced in islet beta cells, and not in the alpha cells, has been documented by immunohistochemical staining of islet sections and by Western blot analysis of cell-sorted cytokine exposed islets [78, 79]. Interestingly, whereas LPS + TNFα induce iNOS expression in intact isolated rat islets (as in macrophages), they do not induce iNOS expression in isolated alpha or beta cells. In contrast, IL-1β alone induces iNOS in both intact islets and isolated beta cells, but not in alpha cells [79], suggesting that LPS and TNFα stimulate intra-islet IL-1β release, which subsequently induces iNOS expression in the beta cells, resulting in impaired function [79].

In both rodent insulin-producing cell lines and in rat islets IL-1β alone is sufficient for induction of iNOS mRNA expression [18, 27, 77]. Both TNFα and IFNγ potentiate IL-1β-induced iNOS mRNA expression [69, 80], and TNFα + IFNγ are more effective than IL-1β in increasing beta-cell mRNA

content [69, 81]. TNFα alone induces a low level of iNOS expression and NO production in insulin-producing RIN cells, but neither IFNγ nor LPS by themselves induce iNOS activation in these cells [69, 80]. However, IFNγ increases NO production by mouse pancreatic islets, similar to the values observed with IL-1β [80], an effect which may be mediated by non-beta cells present in the islets. In human pancreatic islets a combination of two (IL-1β + IFNγ) or three (IL-1β + IFNγ + TNFα) cytokines is required for iNOS mRNA expression [8] and NO production [7, 8]. As described for other cell types [4], iNOS mRNA expression in beta cells is mainly regulated at the transcriptional level. This is suggested by the following observations: a) actinomycin D, a blocker of gene transcription, prevents IL-1β-induced iNOS expression in insulin producing cells [70, 81, 82] and NO production by adult rat islets [19] b) IL-1-β increases iNOS mRNA expression in RIN cells without affecting mRNA stability (total cellular mRNA content results from a balance between mRNA transcription and degradation, and the lack of effects of IL-1β on iNOS degradation indirectly suggests that the cytokine increases iNOS transcription) [69]. iNOS mRNA expression induced by cytokines in insulin-producing cells is also suppressed by cycloheximide [70, 81, 82], by high concentrations of nicotinamide (50 mmol/l, an effect probably mediated by inhibition of protein synthesis [77]) and by cAMP generators [83, 84]. These inhibitory effects of cycloheximide (20 μg/ml) on iNOS mRNA cannot be explained by non-specific deleterious effects of the drug, since cycloheximide neither inhibits glyceraldehyde-3-phosphate dehydro genase (GAPDH) mRNA expression [70, 81, 82] nor prevents IL-1-induced manganese superoxide dismutase (MnSOD) mRNA expression [127] (MnSOD is expressed in parallel with iNOS following IL-1 exposure; see below). Both lower concentrations of nicotinamide (10–20 mmol/l) and dexamethasone decrease NO production without affecting RIN-cell iNOS mRNA content [77]. Interestingly, IL-4 and IL-10, two cytokines known to prevent iNOS expression in macrophages [85, 86], fail to do so in insulin-producing cells ([77, 87]; for a detailed list of agents which modify NO production by beta cells see [18]). Another potential "suppresser" cytokine in this context is TGFβ, previously shown to prevent iNOS activation and NO production by rodent macrophages [68], rat cardiac myocytes [88] and human retinal pigment cells [89]. Regarding beta cells, preliminary data suggest that islet pre-treatment (18 h) with TGFβ decreases IL-1-induced NO production without affecting iNOS enzyme activity [90]. However, Hao and Palmer [91] did not observe an inhibitory action of TGFβ on the IL-1β actions on rat pancreatic islets. We have also examined the effects of different TGFβ preparations on RIN cells and adult rat islets, and failed to observe inhibitory effects of the peptide on NO production, even when it was added 18–24 h before addition of IL-1β. Moreover, TGFβ also failed to prevent NO production by human islets exposed to IL-1β + IFNγ + TNFα (Eizirik and Flodström, unpublished data). Clearly, further studies are required to establish whether TGFβ indeed modifies iNOS activity in beta cells.

IL-1-induced signal transduction and iNOS activation: role of NF-κB, SAPK/JNK, ceramide and PKC. Interleukin-1 receptor (IL-1R). Several investigators have presented indirect evidence for the presence of IL-1Rs on islet cells [92–97]. More recently, islet-cell receptors for IL-1 were directly visualized and characterized in NOD mice [98]. High affinity IL-1Rs are present at a high density in islet cells of control and pre-diabetic NOD mice, suggesting a physiological role for IL-1 in islet-cell function. Moreover, the density of the receptors decreased sharply with beta-cell loss and the outbreak of diabetes, indicating that beta cells are the main source of islet IL-1R [98]. Although insulin-producing cells express mRNAs for both type I and type II IL-1 receptors [70], type I IL-1R is the main mediator of the biological effects of IL-1 in these cells, as suggested by the observations that an IL-1R antagonist protein (mostly directed against the type I IL-1R) [94] and a monoclonal antibody against type I IL-1R [95] prevented the suppressive effects of IL-1β and IL-1α on rat and mouse islet function. The type I IL-1R is a type IV cytokine receptor and it is unique in that no other known cytokine receptors share extended similarities in overall structure [99]. The extracellular portion of IL-1R consists of three Ig-like domains and the cytoplasmic part is 213 amino acids long. Most other cytokine receptors have either intrinsic domains with phosphotyrosine kinase activity or binding sites for non-receptor phosphotyrosine kinases. For example, the activated and dimerized IFN-γ receptor cross-phosphorylates the two tyrosine kinases Jak1 and Jak2, leading to phosphorylation of the transcriptional activator Stat1 ([99]; Fig. 2). However, this does not seem to be the case for the IL-1R. Instead, IL-1R appears to associate with a putative serine/threonine protein kinase, which is activated in response to IL-1 [100–102].

IL-1-induced signal transduction is today considered to involve at least three major signalling pathways, namely the transcription factor NF-κB, the stress-activated protein kinases (SAPK/JNK) and PKC (Fig. 2). These three pathways seem to activate specific sets of transcription factors that may interact in inducing iNOS and other genes in insulin-producing cells.

Nuclear factor κB (NF-κB). NF-κB is present in most cells, acting mainly as an early immune and inflammatory response mediator [103]. It is activated by a

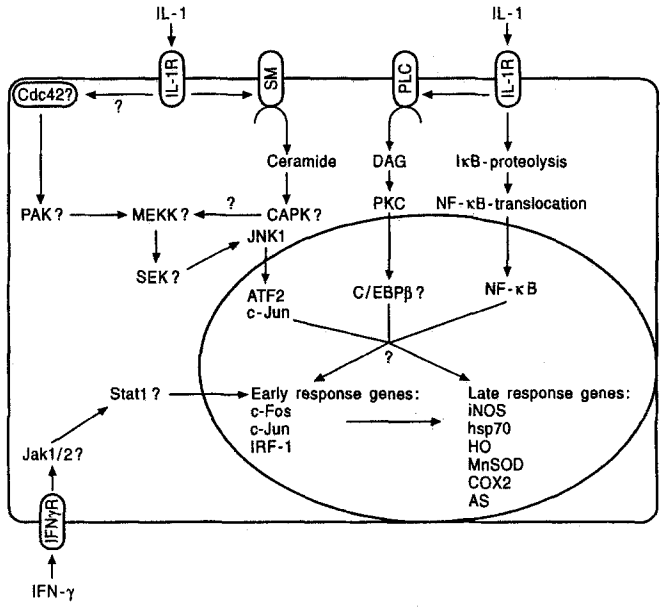

Fig. 2. Schematic illustration of IL-1 and IFN-γ induced signal transduction pathways in insulin-producing cells. IL-1 receptor (IL-1R) activation leads to sphingomyelinase (SM) activation and ceramide generation. This will presumably activate the ceramide-activated protein kinase (CAPK), which increases the activity of c-Jun NH_2-terminal kinase (JNK1). In other cells, IL-1 is known to also activate JNK1 by an alternative pathway starting out with the GTP-binding protein Cdc42 followed by a phosphorylation cascade (PAK, MEKK and SEK). It is, however, unclear whether this pathway operates in insulin-producing cells. JNK1 activation leads to phosphorylation and activation of the transcription factors ATF2 and c-Jun. IL-1 probably also induces a phosphatidylcholine specific phospholipase C (PLC), diacylglycerol (DAG) generation and protein kinase C (PKC) activation. PKC is known to stimulate the microtubulin associated protein (MAP) kinases, which might activate the transcription factor C/EBPβ. IL-1 binding to the IL-1 receptor induces also NF-$\varkappa$B activation, presumably by promoting I$\varkappa$B-proteolysis. Finally, in other cells, IFNγ receptor activation is known to enhance the activities of the protein tyrosine kinases Jak1 and Jak2 and the transcriptional regulator Stat1. The transcription factors ATG2, c-Jun, C/EBPβ, NF-$\varkappa$B and Stat1 probably interact in inducing early response genes (30–60 min) and late response genes (3 h). Some of the early response genes may also participate in the induction of late response genes. Question marks indicate pathways which have not been characterized in insulin-producing cells. Hsp, Heat shock protein70; HO, haem oxygenase; MnSOD, manganese superoxide dismutase; COX2, inducible form of cyclooxygenase; AS, argininosuccinate synthetase

multitude of factors such as viruses, bacteria, oxidative stress and cytokines, and induces the transcription of genes encoding defence and signalling proteins. NF-$\varkappa$B is sequestered in the cytosol of non-stimulated cells as an inactive trimer consisting of p50, Rel-A (p65) and I$\varkappa$B. Upon activation, I$\varkappa$B is phosphorylated and proteolysed, which leads to the release and translocation of p50 and Rel-A dimer to the nucleus where it binds to the B motif (a decameric DNA sequence motif [104]). Interestingly, interaction between a specific cytoplasmic IL-1R domain (residues 508–521) and the IL-1R-associated protein kinase seems to be necessary for NF-$\varkappa$B activation [101]. It is, however, unclear whether the IL-1R associated protein kinase phosphorylates I$\varkappa$B directly or whether other protein kinases are involved.

We observed that in rodent insulin-producing cells IL-1β induces a rapid translocation of NF-$\varkappa$B from the cytosol to the nucleus, a process prevented by the protease inhibitor tosyl-L-lysine chloromethylketone (TLCK) [105]. TLCK has been shown to prevent IL-1-induced inhibition of islet function [106] and proteolysis of I$\varkappa$B in other cell systems [107]. TLCK and the NF-$\varkappa$B inhibitor pyrrolidine dithiocarbamate (PDTC) also prevented iNOS expression in insulin-producing cells, suggesting that NF-$\varkappa$B activation is necessary for IL-1β-induced NO production [81, 105, 108]. These observations were later reproduced by Kwon et al. [109]. PDTC is an antioxidant and it is therefore thought to counteract oxygen free radical-induced activation of NF-$\varkappa$B [110]. Further findings in support of a role of oxygen free radicals in IL-1-induced activation of NF-$\varkappa$B and iNOS are: a) hydrogen peroxide addition to rat [111] and human (Mello and Eizirik, unpublished data) islet cells increased production of nitrite (nitrite is a stable product of NO reaction with molecular oxygen) b) the radical scavengers N-acetyl cysteine [109] and ebselen (Mello, Flodström and Eizirik, unpublished data) prevented NF-$\varkappa$B activation and iNOS expression in RINm5F cells. On the other hand, we have recently observed that the addition of 100 or 300 μmol/l of hydrogen peroxide to RINm5F cells did not induce NF-$\varkappa$B translocation in gel shift experiments (Fig. 3). Moreover, when using the fluorescent probe dichlorofluorescein diacetate, which is activated intracellularly and oxidized to its fluorescent state by hydrogen peroxide [112], we could not observe any increase in the generation of hydrogen peroxide in response to IL-1β addition (data not shown). Clearly, further studies are warranted to determine whether oxygen free radicals mediate IL-1 induced NF-$\varkappa$B activation and iNOS expression.

As mentioned above, the presence of IL-1β alone is enough to induce iNOS transcription and NO formation in rat islets, while three cytokines (IL-1β + TNF-α + IFN-γ) are required for iNOS mRNA expression in human pancreatic islets [8]. Thus, it has been unclear whether NF-$\varkappa$B is also of relevance for NO production by human islets. We have recently observed by electrophoretic mobility shift assay (EMSA) that NF-$\varkappa$B is activated in human islets treated with the three cytokines and that this increase is completely prevented by PDTC [113]. PDTC also inhibited cytokine-induced nitrite formation, indicating that NF-$\varkappa$B plays an important role in the induction of iNOS transcription in human islets. However, IL-1β alone enhanced NF-$\varkappa$B activity but failed to induce nitrite production in these cells [113]. These

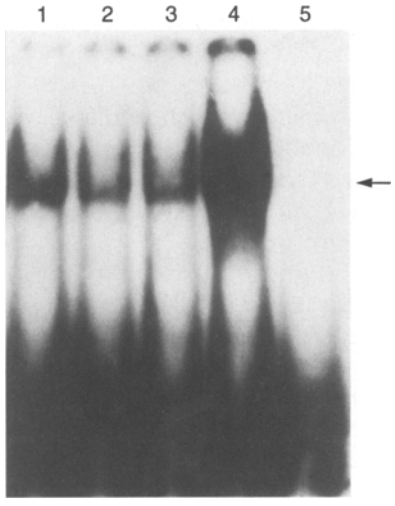

Fig. 3. Gel shift analysis of NF-$\varkappa$B binding in nuclear extracts from RINm5F cells exposed to 100 μmol/l hydrogen peroxide (lane 2), 300 μmol/l hydrogen peroxide (lane 3) and 25 U/ml IL-1β (lane 4) for 20 min. Lane 1 is control and lane 5 is negative control using 100 fold excess of unlabelled oligonucleotide. Representative of three separate experiments

observations suggest that NF-$\varkappa$B activation is necessary but not sufficient to evoke iNOS expression in human pancreatic islets. Thus, it is conceivable that other *trans*-acting factors besides NF-$\varkappa$B are required for iNOS transcription in these cells. We have shown that cytokine-induced iNOS mRNA transcription in RINm5F and HIT-cells is inhibited by cycloheximide [70, 81, 82], a blocker of protein synthesis. Activation of NF-$\varkappa$B is not dependent on the synthesis of new proteins [103] implicating a role for other factor(s) in the signal cascade. Recently, it was shown that macrophages from knock-out mice defective in the interferon response factor-1 (IRF-1) do not produce NO in response to immunostimulants [114]. This factor, which requires de novo synthesis, has recently been suggested to play a role in IL-1β-induced iNOS expression in rat islets [115]. Considering that there are sequences for binding of IRF-1 both in the rodent and human iNOS promotor region (Fig. 1), it is conceivable that concomitant activation of NF-$\varkappa$B and IRF-1 (and possibly other *trans*-acting factors) are needed to induce iNOS transcription. One possibility is that while IL-1β activates both NF-$\varkappa$B and IRF-1 in rodent islets, it activates only NF-$\varkappa$B in human islets. If both factors are indeed necessary for iNOS expression, this could explain why two or three cytokines are required for iNOS induction in human islets, i.e. IL-1β and TNFα as activators of NF-$\varkappa$B, and IFNγ as activator of IRF-1.

Another transcription factor of potential relevance for iNOS activation is AP-1. The murine iNOS gene promoter contains two copies of the AP-1 binding motif [57]. IL-1β induces an early activation of c-*fos* mRNA in insulin-producing cells [70, 116], preceding iNOS mRNA expression [70, 108]. The protein product of c-*fos* forms a protein complex with the product of c-*jun* (AP-1 complex), activating genes with AP-1 binding sites in their promoter region [117]. However, AP-1 activation by itself is not sufficient for iNOS activation in insulin-producing cells, since PDTC increases c-*fos* expression without affecting iNOS mRNA [108], and phorbol ester failed to induce iNOS [27].

SAPK/JNK. The stress activated/c-Jun NH_2-terminal protein kinases (SAPK/JNK) belong to the mitogen-activated protein kinase (MAPK) superfamily and are known to be activated by IL-1, TNFα, heat, arsenite, ultraviolet-light, osmotic and chemical stress in different cell types [118]. SAPK/JNK are activated by a linear cascade starting with the GTP-binding proteins Cdc42/Rac-1 followed by the postulated protein kinase PAK, the recently characterized MEK kinase, SEK and finally SAPK/JNK [119]. This pathway is homologous to the traditional MAPK pathway, which is activated by tyrosine kinase receptors and tyrosine kinase-linked receptors that interact with the GTP-binding protein Ras [120]. SAPK/JNK has been shown to phosphorylate the small heat shock proteins (hsp) [121] and the transcription factors c-Jun and ATF2 [122, 123], leading to different stress responses, growth arrest or apoptosis [124]. SAPK/JNK activation has been shown to be induced by the lipid ceramide in different cell types [125]. Ceramide is cleaved off from sphingomyelin by the plasma membrane enzyme sphingomyelinase, a process which occurs in response to IL-1 or TNF-α addition. It has been proposed that ceramide stimulates a ceramide-activated protein kinase (CAPK) which might stimulate SAPK/JNK activity at some unknown level [126]. We have recently observed that IL-1 induces sphingomyelinase activity and ceramide generation in RINm5F cells [127]. Moreover, addition of a ceramide analogue modestly increased the activity of JNK1, leading to phosphorylation of the transcription factors ATF2 and c-Jun [127]. This effect was paralleled by an enhanced binding in nuclear extracts to the ATF/CRE element as determined by gel shift analysis. Thus, IL-1-mediated ceramide generation appears to activate the SAPK/JNK pathway. However, the effects of the ceramide analogue were weaker than those evoked by IL-1, indicating that activation of the Cdc42-dependent pathway is also necessary to obtain a full effect. Interestingly, the ceramide analogue did not induce NF-$\varkappa$B activation or NO production, indicating that there is no cross-talk between the ceramide and NF-$\varkappa$B pathways in insulin-producing cells [127]. Moreover, the ceramide analogue failed to induce iNOS and MnSOD mRNA expression in RINm5F cells ([127]; Bedoya, Flodström and Eizirik, unpublished data), suggesting that ceramide generation is not sufficient for iNOS induction.

PKC and tyrosine protein kinases. We have recently observed that IL-1β stimulates the generation of diacylglycerol (DAG) both in isolated mouse islets and RINm5F cells [127, 128]. This effect was not paralleled by an increase in intracellular free Ca^{2+} concentrations, suggesting that IL-1β activates a phosphatidylcholine specific phospholipase C. The increased DAG content resulted in an enhanced phosphorylation of the 87 kDa PKC substrate [128]. Activation of PKC might explain the stimulatory effect of IL-1β on islet insulin release observed during the first 1–2 h after cytokine addition, since PKC is a well-known potentiator of glucose-induced insulin release [129]. In other cell types PKC is known to interact with the MAPK and the NF-$\varkappa$B pathways. In RINm5F cells, however, there was only a weak and non-significant effect of the synthetic PKC activator phorbol 12-myristate (PMA) on NF-$\varkappa$B translocation [127], and no effect of the phorbol ester on iNOS mRNA expression [27]. On the other hand, the finding that PMA evoked a modest increase in JNK1 activity might indicate that PKC interacts with the SAPK/JNK pathway [127]. This cross-talk might be the result of PKC activation of sphingomyelinase, since DAG is known to trigger sphingomyelinase activity in Jurkat T cells and in GH3 rat pituitary cells [130, 131]. If PKC has only weak effects upon the NF-$\varkappa$B and the SAPK/JNK pathways, then which are its main targets in beta cells? It has been reported that PKC activation leads to the phosphorylation of the transcription factor C/EBPβ, an effect probably mediated by the traditional MAP kinases [132]. C/EBPβ is a transcriptional activator present in differentiated cells, such as hepatocytes, and is known to be involved both in the acute-phase response and in IL-1-induced gene expression [133]. We are currently investigating whether there is a role for C/EBPβ in IL-1 induced signal transduction, and preliminary experiments indicate that IL-1 induces phosphorylation of C/EBPβ in RINm5F cells (Saldeen and Welsh, unpublished data). Interestingly, C/EBP binding sites have recently been demonstrated in the haem oxygenase-1 enhancer region and these elements seem to enhance oxidative stress-induced expression of this gene [134]. Thus, it may be that PKC and C/EBPβ participate in the induction of defence/repair responses following beta-cell exposure to cytokines (see below).

It has been reported that inhibitors of tyrosine protein kinases modulate IL-1-induced iNOS expression in insulin-producing cells [78, 135], probably by blocking nuclear translocation of NF-$\varkappa$B [109]. Interestingly, tyrosine kinase inhibitors can also prevent IL-1-induced iNOS expression in rat mesangial cells without affecting NF-$\varkappa$B activation [136]. However, since high concentrations of the inhibitors were required to prevent IL-1-induced NO production in insulin-producing cells and since the inhibitors are not completely specific, it remains to be demonstrated whether tyrosine kinases are directly involved in IL-1 induced signal transduction. Indeed, we have been unsuccessful in demonstrating differences in tyrosine phosphorylation of proteins from RINm5F cells exposed to IL-1β for 0, 2, 10 and 30 min using immunoblot analysis (Welsh, unpublished data).

Other late response genes induced by cytokines in pancreatic islets

In parallel with the expression of iNOS, cytokines induce several other genes and proteins in pancreatic islets (Fig. 2). Indeed, 24-h culture of neonatal rat islets in the presence of IL-1β upregulates 29 proteins, as judged by high-resolution two-dimensional gel electrophoresis [137]. The identity of most of these proteins remains unknown, but available data suggest that some may contribute to NO production and NO-induced beta-cell dysfunction, while others are involved in beta-cell defence and repair. Among the former, two deserve special attention: firstly, the mRNA encoding for the enzyme argininosuccinate synthetase (AS), and secondly the gene encoding for the inducible form of cyclooxygenase (iCOX).

Arginine is the only known physiological substrate for NO formation, and both in macrophages [138] and in rodent and human islets arginine availability affects cellular rates of NO synthesis ([81]; Flodström, Morris and Eizirik, unpublished data). This is relevant for the in vivo situation, where plasma L-arginine concentrations (80–100 μmol/l) are normally below the K_m for macrophage iNOS (0.15 mmol/l arginine; [138, 139]). Arginine may decrease even further during sepsis and at inflammatory sites, probably due to arginase release from activated macrophages [140]. Citrulline, the by-product of iNOS reaction, can be recycled to arginine by the action of AS and argininosuccinate lyase [138]. In vitro induction of iNOS in macrophages [73], smooth muscle cells [141] and insulin-producing cells [81], and in vivo induction of iNOS in lung and spleen [142], is paralleled by induction of mRNA and enzyme activity for AS, the rate-limiting step in this reaction. Furthermore, both adult rat islets treated with IL-1β and human pancreatic islets exposed to IL-1β + IFNγ + TNFα utilize citrulline to regenerate arginine and produce NO, confirming the physiological relevance of this pathway ([81]; Flodström, Morris and Eizirik, unpublished data). A detailed analysis of cytokine-induced AS mRNA expression in insulin-producing RIN cells [81] showed a similar time course for AS and iNOS expression, with maximal mRNA content after 6 h in the presence of IL-1β or TNFα + IFNγ. IL-1β-induced increase in AS mRNA is independent of NO generation, since it is not prevented by inhibitors of iNOS activity or reproduced by chemical NO

donors. Cytokine-induced expression of iNOS and AS mRNAs are prevented by actinomycin D, cycloheximide and PDTC [81], suggesting that induction of these genes is co-regulated, and depends on a similar signal-transduction culminating in NF-$\varkappa$B activation. It remains to be clarified whether NF-$\varkappa$B response elements are present in the AS promoter/enhancer, as has been described for iNOS (see above). Alternatively, NF-$\varkappa$B activation by cytokines may trigger subsequent cellular events which then lead to AS transcription. The observation that protein synthesis is necessary for induction of AS mRNA is in line with this hypothesis.

In murine macrophages LPS induces in parallel iNOS and arginase activity [143]. Arginase (Type II) produces L-ornithine and urea from arginine. Since iNOS and arginase utilizes arginine as substrate, arginase may decrease NO formation by competing for the amino acid. When adequate precursors are provided, rat pancreatic islets can produce urea, but the urea cycle is not operative in these cells due to lack of ornithine transcarbamylase activity [144]. If cytokines indeed induce iNOS and arginase in parallel in beta cells, this could lead to both urea production and decreased NO formation. However, RINm5F cells exposed to IL-1β (both in the absence or presence of iNOS inhibitors) do not produce urea (Flodström and Eizirik, unpublished data), suggesting lack of arginase activation. This may be due to inhibition of arginase by N^{ω}-hydroxy-L-arginine, an intermediate in the L-arginine-nitric oxide pathway shown to inhibit the enzyme activity in liver cells and macrophages [145].

Arachidonic acid and its metabolites are important mediators of the inflammatory response. The oxidation of arachidonic acid is mediated by two main pathways: 1) the cyclooxygenase pathway, leading to prostaglandins and thromboxane formation; 2) the lipoxygenase pathway, leading to the synthesis of leukotrienes. It is of note that both pathways are stimulated by IL-1 in pancreatic islets. Thus, IL-1β induces synthesis of the inducible form of cyclooxygenase (iCOX) in rat pancreatic islets, leading to increased prostaglandin E_2 (PGE_2) formation [146]. Actinomycin D prevented IL-1β-induced iCOX and iNOS protein synthesis, indicating that RNA transcription is required for expression of iCOX. N^G-monomethyl-L-arginine, a competitive inhibitor of iNOS activity, significantly decreased IL-1β-induced PGE_2 formation in rat islets without inhibiting iCOX synthesis, suggesting that NO directly activates iCOX enzymatic activity [146]. Similar observations were made in mouse macrophages and human fibroblasts [147], raising the possibility that when both iNOS and iCOX are induced by cytokines, there is an NO-mediated increase in the production of pro-inflammatory prostaglandins that may exacerbate the inflammatory response. IL-1 also increases activity of 12-lipoxygenase and consequent synthesis of the product 12-hydroxyeicosatetraenoic acid [148, 149]. However, it remains to be clarified whether this effect is due to IL-1-induced increased expression of 12-lipoxygenase mRNA and protein [148], or whether it is the consequence of increased substrate availability through an NO-dependent mechanism [149].

In vitro data indicate that rodent and human beta cells are able to repair themselves after damage by cytokines [150] and NO donors [26]. These observations indicate that beta cells can activate repair and/or defence mechanisms following exposure to these agents (reviewed in [11]). Among the potential "defence" proteins induced by IL-1β are manganese superoxide dismutase (MnSOD) [151], heat shock proteins (hsp) 70 and 90 [152–154] and haem oxygenase [154–156].

There is indirect evidence suggesting that IL-1β, specially in combination with TNFα and IFNγ, may cause the synthesis of oxygen reactive species, such as superoxide and hydrogen peroxide, in pancreatic islets [12, 13]. Moreover, activated macrophages, usually present in islets during insulitis, produce NO together with the reactive species described above [157]. NO can combine with superoxide to form the potent oxidizing agent peroxynitrite [158], and recent data suggest that peroxynitrite, and not NO itself, is responsible for aconitase inactivation [159, 160] and consequent mitochondrial dysfunction [19]. In this context, MnSOD activation could have a beneficial effect, by removing superoxide and thus decreasing peroxynitrite formation in the vicinity of beta cells. We have shown that IL-1β, or TNFα + IFNγ, induce MnSOD mRNA expression and enzyme activity in both rodent islets [151] and in insulin-producing RIN cells [82, 108], while a combination of three cytokines (IL-1β + IFNγ + TNFα) is required to induce MnSOD protein expression in human pancreatic islets [155]. Induction of iNOS and MnSOD mRNAs by IL-1β has a similar time-course in insulin-producing cells, with increased mRNA levels detectable after 4 h and subsequent increase at 6 h. Aminoguanidine, an iNOS inhibitor, did not prevent IL-1β-induced MnSOD mRNA expression, and sodium nitroprusside, an NO releasing agent, failed to induce MnSOD mRNA, suggesting that increased expression of MnSOD is a direct effect of IL-1β, independent of NO generation [82]. Actinomycin D prevented both IL-1β-induced iNOS and MnSOD mRNA expression, while cycloheximide [82] and PDTC [108] inhibited cytokine-induced iNOS expression without affecting MnSOD expression. Thus, protein synthesis and NF-$\varkappa$B activation are necessary for iNOS, but not for MnSOD, expression in insulin-producing cells. These data suggest that IL-1β signals for the activation of genes that may lead to beta-cell damage (i.e. iNOS) or defence (i.e. MnSOD) by different pathways. This opens the intriguing possibility

of protecting beta cells in early IDDM by agents that allow expression of "protective" effects of cytokines, without the accompanying induction of "deleterious" effects [108].

Hsp70 expression is induced in rat islets by cytokines [152–154], and introduction of bovine hsp70 into rat islets by the liposome technique [161], or induction of endogenous hsp70 by high temperature [162], protected rat islets against IL-1β- or NO-induced dysfunction. Moreover, it seems that high expression of hsp70, together with increased activities of SOD and catalase, make human islets less sensitive than rodent islets to the deleterious effects of cytokines and NO [155]. Unfortunately, little is known about the molecular regulation of hsp70 in beta cells. The same holds true for the antioxidant protein haem oxygenase [154–156].

Future directions

The first paper on cytokine-induced NO formation in pancreatic islets was published in 1990 [17]. As reviewed above, a large amount of data on iNOS regulation in beta cells have been generated during the subsequent 5 years. However, crucial questions remain to be answered. Among them, the exact nature of the signals generated by IL-1β-binding to beta-cell surface receptors; the reasons for the differential iNOS induction in rodent and human islets; the complete sequence of the iNOS promoter regions in human beta cells; the identity of the large group of proteins induced by IL-1 in rodent pancreatic islets; the molecular regulation of AS, iCOX, MnSOD, hsp70 and haem oxidase expression in beta cells. Furthermore, several of the above described in vitro observations must also be tested on in vivo models of IDDM, such as NOD mice and BB rats. In this context, a crucial question is whether turning off the iNOS gene systemically and/or in the beta cell can prevent IDDM. Mice with systemic iNOS (knock-out mice) have already been produced by homologous recombination [163–165], and it is anticipated that in the near future crosses between these mice and NOD mice will provide some answers to these questions.

The concept of the Harmony of the Spheres received great attention in the late Middle Ages and the Renaissance. This theory is derived from Pythagoras, and suggests that heavenly bodies are carried in the space inside spheres. These revolving spheres emitted musical sounds according to their velocity, size and relative distance – the Harmony or Music of the Spheres (for an overview on the subject see [166]). When cytokines bind to surface receptors in the pancreatic beta cells, there is induction of different intracellular signals, leading to transcription of several genes. Some of these genes may be involved in beta-cell damage, while others are probably part of beta-cell repair mechanisms. The integrated activation of these different cytosolic signals, mRNAs and proteins in the beta cells may be seen as a microcosmic Harmony of the Spheres. It is our conviction that once this whole cellular music is unveiled, it may be possible to change the tune, and thus prevent beta-cell destruction in early IDDM.

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