Is there a role for neuronal nitric oxide synthase (nNOS) in cytokine toxicity to pancreatic beta cells?

Ewa Gurgul-Convey*, Katarzyna Hanzelka, Sigurd Lenzen

Institute of Clinical Biochemistry, Hannover Medical School, Hannover, Germany

**ARTICLE INFO**

Article history:
Received 11 April 2012
Revised 17 July 2012
Available online 14 August 2012

Keywords:
Cytokines
Diabetes
Insulin-secreting cells
Islets
nNOS
iNOS

**ABSTRACT**

Nitric oxide (NO), produced by the action of the inducible NO synthase, plays a crucial role in cytokine toxicity to pancreatic beta cells during type 1 diabetes development. It was the aim of this study to analyze the role of the neuronal NOS (nNOS) in proinflammatory cytokine-mediated beta cell toxicity. Expression of different isoforms of nitric oxide synthase in insulin-secreting INS1E cells and rat islets was analyzed by quantitative real-time PCR and Western blotting. The expression of nNOS in insulin-secreting INS1E cells was similar to that found in rat brain, while two other isoforms, namely the endothelial eNOS and inducible iNOS were not expressed in untreated cells. IL-1β alone or in combination with TNF-α and/or IFNγ induced iNOS but not eNOS expression. In contrast, nNOS expression was strongly decreased by the mixture of the three proinflammatory cytokines (IL-1β, TNF-α and IFNγ) both on the gene and protein level in INS1E cells and rat islet cells. The effects of cytokines on glucose-induced insulin-secretion followed the pattern of nNOS expression reduction and, on the other hand, of the iNOS induction. The data indicate that a low level of nitric oxide originating from the constitutive expression of nNOS in pancreatic beta cells is not deleterious. In particular since proinflammatory cytokines reduce this expression. This nNOS suppression can compensate for NO generation by low concentrations of IL-1β through iNOS induction. Thus, this basal nNOS expression level in pancreatic beta cells represents a protective element against cytokine toxicity.

© 2012 Elsevier Inc. All rights reserved.

**Introduction**

Nitric oxide (NO) is a free radical, produced in many cell types under physiological conditions in low concentrations from L-arginine by a constitutive NO synthase and acting as an important signalling molecule [1,2]. There are three genetically different isoforms of NO synthase which can account for NO production. They comprise neuronal NO synthase (nNOS), inducible NO synthase (iNOS) and endothelial NO synthase (eNOS) [1]. Two isoforms, nNOS and eNOS, are expressed constitutively, nNOS preferentially in the brain and eNOS in the endothelium [1]. The activity of both of these isoforms is regulated by intracellular calcium concentration changes [1]. The expression of the iNOS isoform is induced by different cytokines and endotoxin, and is fully activated at basal calcium concentration [1].

Abbreviations: HEPES, 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid; IFNy, interferon gamma; iNOS, inducible nitric oxide synthase; IL-1β, interleukin 1 beta; eNOS, endothelial nitric oxide synthase; ER, endoplasmic reticulum; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; TNF-α, tumor necrosis factor alpha.
* Corresponding author. Address: Institute of Clinical Biochemistry, Hannover Medical School, Carl-Neuberg-Str. 1, 30625 Hannover, Germany. Fax: +49 5115323584.
E-mail address: Gurgul-Convey.Ewa@mh-hannover.de (E. Gurgul-Convey).

The neuronal isomorf is the predominant source of NO in neurons, but it is also expressed in skeletal muscle, heart and smooth muscle, where NO controls blood flow and muscle contractility [1]. The nNOS enzyme is localized in the cytoplasmic, mitochondrial or nuclear compartment of the cell, displaying diverse intracellular functions depending on its localization [1]. The activity of nNOS can be modulated on different levels, e.g. by calmodulin binding, dimerization, protein–protein interactions or phosphorylation [1].

Reactive oxygen and nitrogen species can impair the function of a variety of biomolecules and lead to dysfunction of cellular organelles, thereby causing various disorders [3–7]. The cytokine-mediated iNOS induction together with an excessive NO generation in pancreatic beta cells is a crucial element of cytokine toxicity during type 1 diabetes development [4,7,8]. In insulin-secreting cells NO is a partner molecule for two important chemical reactions occurring during exposure to proinflammatory cytokines and yielding toxic end products. On the one hand, NO can react with superoxide radicals resulting in production of peroxynitrite [7]. On the other hand, the reaction between NO and hydrogen peroxide in the presence of trace metals forms hydroxyl radicals [7]. Although both reactions take place in pancreatic beta cells exposed to cytokines, only the latter one is responsible for cytokine-induced beta cell destruction and death [7]. Moreover, NO is also involved in
cytokine-induced ER stress in insulin-secreting cells [8,9] and the inhibition of insulin secretion [10–12].

Pancreatic beta cells express the neuronal, constitutive isoform of NOS (nNOS) [13]. It resides mainly in secretory granules and has been shown to modulate insulin secretion [13]. Recently Bachar et al. [14] proposed that nNOS may protect pancreatic beta cells from glucototoxicity-induced ER stress and apoptosis. Because the influence of cytokines on nNOS expression in insulin-secreting cells remains unknown, the aim of this study was to analyze the effects of IL-1β, TNF-α and IFN-γ on nNOS.

Materials and methods

Cell culture and cytokine incubation

Insulin-secreting INS1E cells were cultured as described [15,16]. Concentrations for 24 h incubation with cytokines (all from Promocell, Heidelberg, Germany) were: 60 U/ml of IL-1β (4.4 ng/ml); 600 U/ml of IL-1β (44 ng/ml); 185 U/ml of TNF-α (8.7 ng/ml); 1850 U/ml of TNF-α (87 ng/ml); 14 U/ml of IFN-γ (10.3 ng/ml); 140 U/ml of IFN-γ (103 ng/ml). All chemicals used in this study were from Sigma.

Rat tissue isolation and treatment

Pancreatic islets and other tissues were isolated from 250 to 300 g adult male Lewis rats (70–80 days old). Islets were isolated by collagenase digestion and handpicked under a stereo microscope. Isolated islets were cultured overnight in RPMI-1640 medium containing 5 mmol/l glucose, 10% FCS, penicillin, and streptomycin at 37 °C in a humidified atmosphere of 5% CO2 as described before [17]. The islets were then treated with 600 U/ml IL-1β or a mixture of the three proinflammatory cytokines for 24 h following RNA extraction.

RNA isolation, cDNA preparation and real-time PCR

RNA isolation, cDNA production and quantitative real-time PCR measurements were performed as described [9]. The primers used in this study are shown in Table 1. Data were normalized to β-actin. The primers were designed to detect the following sequences: eNOS 73–241 bp (containing the NcoI restriction site), iNOS 1101–1192 bp (containing the Ncol restriction site) and nNOS 375–506 bp (no restriction sites). Restriction analysis was performed using the Aval and the NcoI restriction enzymes (Fermentas) according to the manufacturer’s protocol. DNA electrophoresis was done in 1% agarose gels.

iNOS and nNOS Western blot analyses

INS1E cells were incubated for 24 h with cytokines, washed with ice-cold PBS and homogenized using short bursts (Braun-Sonic 125 Homogenisator, Quigley-Rochester, Inc., Rochester, NY, USA). Protein content was determined by the BCA assay (Pierce, Rockford, IL, USA). 40 μg of total protein was resolved in SDS polyacrylamide gel electrophoresis and then electroblotted onto membranes. Immunodetection was performed using specific primary antibodies against nNOS (catalog number 4234, Cell Signalling; overnight incubation), iNOS (sc-650, overnight incubation) or β-actin (sc-1619, 2 h incubation) (both from Santa Cruz Biotechnology, Heidelberg, Germany) followed by exposure to secondary peroxidase-conjugated AffiniPure antibodies (Dianova, Hamburg, Germany). The immunoreactive bands were visualized through chemiluminescence using the ECL detection system and the INTAS® chemiluminescence detection system (Intas Science Imaging Instruments GmbH, Göttingen, Germany).

Insulin secretion and content

Insulin secretion and content in insulin-secreting INS1E cells were measured by radioimmunoassay (RIA) [18]. Cells were seeded at a density of 350,000 cells/well onto 6-well plates 2 days before the addition of cytokines. A 24-h incubation with cytokines was followed by a 1-h incubation without glucose and a 2-h stimulation with 10 mM glucose. Thereafter, samples were collected for RIA. Insulin values were normalized to the DNA content of the incubated cells.

Immunofluorescence

For immunofluorescence staining INS1E cells were seeded onto collagen-coated glass slides and incubated for 24 h with a cytokine mixture as described above following an overnight fixation with 4% paraformaldehyde in PBS. After fixation cells were washed three times with PBS for 5 min. After a 20 min blocking in PBS with 0.1% Triton X-100 and 1% bovine serum albumin (BSA) at room temperature the cells were washed again as above. The slides were incubated with primary antibodies diluted in PBS with 0.1% Triton X-100 and 0.1% BSA at room temperature for 1 h (rabbit polyclonal anti-nNOS 1:200 (catalog number 4234, Cell Signalling), or guinea pig polyclonal anti-insulin 1:100 (catalog number AB7842, Abcam, Cambridge, UK)) and then washed three times with PBS. The cells were incubated with secondary antibodies (FITC-conjugated anti-rabbit IgG 1:200, or Texas red-conjugated anti-guinea pig IgG 1:200) for 1 h at room temperature (all from Dianova, Hamburg, Germany). For nuclear counterstaining, 300 nM 4-6-diamidino-2-phenylindole (DAPI) was used for 5 min at room temperature. Slides were thereafter mounted with Mowiol (Merck, Darmstadt, Germany). Images were captured (60× oil objective) and analyzed using a CellR®/Olympus IX81 inverted microscope system (Olympus, Hamburg, Germany).

Data analysis

Analyses of the real-time RT-PCR data were performed using the Opticon Monitor v. 1.07 software (MJ Research Inc., Waltham, MA, USA). All data are expressed as means ± SEM. Statistical analyses for each group were performed using the Prism analysis program (Graphpad, San Diego, CA, USA), the Kruskal–Wallis nonparametric test was employed. P values <0.05 were considered statistically significant.

Results

eNOS, iNOS and nNOS transcript expression

To assess the gene expression levels of different NOS isoforms in insulin-secreting INS1E cells and rat islets specific primers for each gene were designed. The specificity of the primers was assured by a choice of sequences of the lowest identity between isoforms.
The analysis of melting curves as well as the end products of real-time PCR reactions for each primer set clearly showed a specific product formation (Fig. 1B–D). The appearance of specific products was also confirmed using positive controls (for eNOS: rat lung, for nNOS: rat brain, Fig. 1C). Noteworthy the restriction analysis of the real-time PCR products for each primer set revealed the specificity of the primers used (Fig. 1D).

Quantitative real-time PCR analysis of gene expression of eNOS, iNOS and nNOS in insulin-secreting INS1E cells revealed a significant nNOS expression, similar to that found in rat brain, and no expression of the two other isoforms. In contrast to INS1E cells pancreatic islets expressed the eNOS isomorf (Fig. 1C). None of the used cytokines induced eNOS transcription in INS1E cells (data not shown). Transcription of iNOS, however, was dramatically increased upon exposure to IL-1β (both by 60 U/ml as well as by 600 U/ml) and its combinations with TNF-α and/or IFNγ (Fig. 2A), confirming earlier observations [9]. In contrast, IL-1β influenced nNOS transcription only mildly (Fig. 2B), whereas there was no effect at all by TNF-α or IFNγ alone (Fig. 2A), confirming earlier observations [9]. In contrast, IL-1β influenced nNOS transcription only mildly (Fig. 2B), whereas there was no effect at all by TNF-α or IFNγ alone (Fig. 2A), confirming earlier observations [9].

IL-1β-dependent pattern of iNOS expression similar to the gene expression was observed on the protein level (Fig. 3A). High concentrations of TNF-α (1850 U/ml) as well as of IFNγ (140 U/ml) alone were ineffective with respect to iNOS induction (Fig. 3A) in the same way as the 10 times lower concentrations of the two proinflammatory cytokines (data not shown). The densitometric analysis of the blots revealed a 5-fold induction of iNOS protein by 60 U/ml IL-1β and a 6-fold induction by 600 U/ml IL-1β (Fig. 3A). No significant additive effects of the other two tested proinflammatory cytokines TNF-α or IFNγ were observed. The incubation with a mixture of all three cytokines led to a massive 12-fold increase in iNOS protein expression (Fig. 3A). TNF-α as well as IFNγ alone did not induce iNOS protein expression, what suggests that the observed weak iNOS gene transcription led to a weak translation providing extremely low levels of iNOS protein. INS1E cells expressed the nNOS protein as shown in Fig. 3B. The nNOS protein expression was decreased in the same fashion as on the transcriptional level, however to a lesser extent (Fig. 3B). The mixture of the three proinflammatory cytokines IL-1β, TNF-α and IFNγ caused a significant, nearly 50% reduction of nNOS protein expression as determined by quantitative densitometric analysis (Fig. 3B) and confirmed by immunofluorescence staining (Fig. 4).

Thus, the translation fashion of eNOS, iNOS and nNOS proteins correlates very well with the transcription pattern of the respective genes.

**nNOS expression in rat islets**

The expression level of nNOS in rat islets was lower than in INS1E cells (Fig. 1C) in line with earlier studies [13]. Exposure to
Fig. 2. iNOS and nNOS gene expression in insulin-secreting INS1E cells after exposure to cytokines. Insulin-secreting INS1E cells were seeded onto 6-well plates at a density of 500,000 cells/well and allowed to attach for 24 h. Thereafter various combinations of cytokines were added (60 U/ml IL-1β, 600 U/ml IL-1β, 60 U/ml IL-1β + 185 U/ml TNF-α, 60 U/ml IL-1β + 14 U/ml IFN-γ, 60 U/ml IL-1β + 185 U/ml TNF-α + 14 U/ml IFN-γ, 1850 U/ml TNF-α, 140 U/ml IFN-γ, 185 U/ml TNF-α + 14 U/ml IFN-γ). After a 24 h-incubation samples were collected. (A) iNOS gene expression (arbitrary units); (B) nNOS gene expression (arbitrary units). Shown are means ± SEM from quantitative real-time PCR analysis (n = 12). Data were normalized to β-actin. *p < 0.05, **p < 0.01, ***p < 0.001 vs. untreated. ANOVA followed by a nonparametric Kruskal–Wallis test.

Fig. 3. iNOS and nNOS protein expression in insulin-secreting INS1E cells after exposure to cytokines. Insulin-secreting INS1E cells were seeded onto 6-well plates at a density of 500,000 cells/well and allowed to attach for 24 h. Thereafter various combinations of cytokines were added (60 U/ml IL-1β, 600 U/ml IL-1β, 60 U/ml IL-1β + 185 U/ml TNF-α, 60 U/ml IL-1β + 14 U/ml IFN-γ, 60 U/ml IL-1β + 185 U/ml TNF-α + 14 U/ml IFN-γ, 1850 U/ml TNF-α, 140 U/ml IFN-γ, 185 U/ml TNF-α + 14 U/ml IFN-γ). After a 24 h-incubation samples were collected. (A) iNOS protein expression; (B) nNOS protein expression. Shown are representative blots and quantitative densitometric analyses of 8 independent experiments (means ± SEM, arbitrary units, normalized to β-actin). *p < 0.05, **p < 0.01, ***p < 0.001 vs. untreated. ANOVA followed by a nonparametric Kruskal–Wallis test.
IL-1β or a cytokine mixture (IL-1β, TNF-α and IFN-γ) caused a significant reduction of nNOS expression (to 38 ± 11% after IL-1β and 18 ± 6% after cytokine mixture compared to the 100% control value, n = 4, p < 0.05).

Glucose-induced insulin secretion and insulin content after cytokine incubation

Insulin secretion in the presence of 10 mM glucose was analyzed after a 24 h exposure of INS1E cells to different cytokines and their combinations (Fig. 5A). The experiments revealed a strong IL-1β-dependent inhibition of glucose-induced insulin secretion, which was slightly potentiated by TNF-α (Fig. 5A). In contrast the incubation of INS1E cells even with high concentrations of IFN-γ did not result in insulin secretion inhibition (Fig. 5A). Moreover, IL-1β significantly reduced insulin content, an effect potentiated by TNF-α, but, again, not by IFN-γ (Fig. 5B).

Discussion

Nitric oxide (NO) produced in low concentrations by constitutive nitric oxide synthases plays a signalling role in different tissues, however an excessive production of NO, driven by iNOS, is thought to underlie the development of various diseases [3–7]. The expression of different NOS isoforms is specifically and tightly regulated by various mechanisms. The expression of iNOS in pancreatic beta cells crucially depends on the activation of the transcription factor NF-κB and, to a lesser extent, on the JAK/STAT pathway [19]. The inhibition of NF-κB signalling via e.g. JANEX-1 [20], antiinflammatory cytokines [21], prostacyclin [22] or some plant extracts [23] prevents cytokine-induced iNOS expression and cytokine toxicity in insulin-secreting cells [1,24,25]. The expression of nNOS is controlled by alternative splicing and the transcription factor CREB [26] and its activity can be regulated by Hsp90, calmodulin and other factors [1,26,27].
The present study showed that in insulin-secreting INS1E cells only the nNOS isoform was expressed constitutively, thus confirming earlier reports [13]. The observed eNOS expression in rat islets originates most likely from glucagon and somatostatin-secreting cells, in which the expression of eNOS was reported earlier [13]. Cytokines significantly reduced nNOS expression both on the transcriptional and translational level. The expression level of nNOS in rat islets was lower, but the results from the rat islet cells treated with cytokines confirmed the data obtained from INS1E cells regarding the decrease of nNOS expression upon exposure to cytokines. Interestingly, the cytokine-mediated changes of nNOS expression closely match the pattern of cytokine toxicity in insulin-secreting cells, which we reported earlier [9]. It is possible that either the induction of pro-apoptotic and/or the reduction of prosurvival pathways may influence the transcriptional control of nNOS expression and/or its translation and activity. As recently shown the activation of the JNK pathway may lead to the reduction of nNOS protein expression [27], therefore the observed decrease in nNOS protein expression after a cytokine mixture treatment may result, at least partially, from the cytokine-induced JNK activation, a well established phenomenon in pancreatic beta cells [28–30]. Other possible regulatory mechanisms could also include changes in the alternative splicing of nNOS mRNA as well as inactivation of nNOS activity, for instance by disturbances in the intracellular calcium pool and Hsp90 availability.

As we have recently shown IL-1β alone or in combination with TNF-α and/or IFN-γ is able to induce a strong rise in NO formation in insulin-secreting INS1E cells, while TNF-α and IFN-γ do not stimulate iNOS and NO production [9]. In contrast to a strong activation of NF-κB by IL-1β [21,31], TNF-α is known to induce NF-κB only mildly [21,31]. Since the transcription factor NF-κB is crucial for cytokine-induced iNOS expression, the observed lack of iNOS induction by TNF-α is not surprising. Moreover, it is also possible that TNF-α may stimulate some alternative signalling pathways which in turn reduce iNOS expression. A significantly higher induction of iNOS expression by the mixture of IL-1β, TNF-α and IFN-γ as compared to IL-1β alone, indicates that in the late stages of islet infiltration during T1DM development, when all three cytokines are produced by activated immune cells in the vicinity of beta cells, insulin-secreting cells face a massive generation of NO. This favors the reaction between NO and the hydrogen peroxide produced in parallel, and leads to formation of highly toxic hydroxyl radicals, which ultimately cause pancreatic beta cell death [6].

The present study showed a strong inhibition of glucose-induced insulin secretion as well as insulin content by IL-1β or its combinations with TNF-α and/or IFN-γ. A slight potentiating effect of TNF-α alone and no influence of IFN-γ were observed. This is in line with earlier reports demonstrating a strong deleterious effect of IL-1β on islet cell function and a weaker effect of TNF-α. It has been shown that this inhibitory effect of IL-1β strongly depends on NO formation derived from iNOS induction [10–12,32–34].

The present study shows that cytokine-mediated NO production in pancreatic beta cells can originate only from the cytokine-induced iNOS expression, and not from any other NOS isoform. The results indicate that a basal level of NO (produced by nNOS) is apparently not deleterious to the beta cells. nNOS has even been shown to prevent iNOS induction in some cell types [35,36] and a partial nNOS knock-down has been shown to result in increased JNK phosphorylation and Chop production, leading to apoptosis [14]. Thus a reduction of nNOS expression by cytokines might be unfavorable for beta cell function. A little NO formation through a mild iNOS induction by low concentrations of IL-1β in insulin-secreting cells might compensate this negative effect. Indeed, it has been shown before that IL-1β in very low concentrations can potentiate glucose-induced insulin secretion [37] rather than causing damage to the beta cells.

We showed that insulin-secreting INS1E cells and rat islets constitutively express nNOS confirming earlier observations [13,38]. INS1E cells are known for their excellent glucose responsiveness [15], which we confirmed in this study. Pancreatic beta cell nNOS is thought to exert two different catalytic activities and the balance between NO production and a nonoxidizing calmodulin-dependent reductase activity is required for the biphasic response of beta cells to glucose stimulation [13]. Moreover, it has been shown that a pharmacological inhibition of nNOS in insulin-secreting INS1E cells and rat islets enhances glucose-induced insulin secretion [13,38]. Therefore the earlier observations together with our present data indicate that the basal nNOS expression plays a regulatory role in insulin secretion, probably by preventing possible episodes of hyperinsulinaemia, but is not disadvantageous for the beta cell function or does not promote beta cell vulnerability. A decreased catalytical activity of nNOS and its relocalization have been shown to be involved in the hyperactivity of beta cells from insulin-resistant rats and also from human islets isolated from obese individuals [39].

The present data strongly indicate that a very small rate of NO formation, derived either from a basal nNOS expression under physiological conditions or from a very weak iNOS expression, which may originate from IL-1β present in beta cells at low concentrations [9], as e.g. in a type 2 diabetes situation [8,40], will not account for beta cell damage and death. The very low IL-1β expression in beta cells in animal models of type 2 diabetes as well as in patients with type 2 diabetes, as reported earlier [40–42], will be insufficient to induce iNOS expression significantly, which would be a prerequisite for NO production that can cause apoptotic beta cell death.

Thus, NO is toxic to beta cells only when generated in high concentrations by cytokine-induced iNOS, as occurs in the type 1 diabetes situation [8]. The constitutive expression of nNOS in pancreatic beta cells, on the other hand, represents rather a protective element against cytokine toxicity, in particular in a type 2 diabetes setting. The present data virtually exclude the concept of a significant role of very low IL-1β concentrations generated in pancreatic beta cells in the type 2 diabetes etiopathology as proposed a number of years ago [41].

Conflict of interest

The authors declare no financial or commercial conflict of interest.

Acknowledgments

This work was supported by a grant from the European Union (Collaborative Project NAIMIT in the 7th Framework Programme, Grant Agreement No. 241447). We would like to thank very much Prof. Claes B. Wollheim (Department of Cell Physiology and Metabolism, University of Geneva, Geneva, Switzerland) for providing insulin-secreting INS1E cells for this study. The excellent technical assistance of Ch. Heinrichs is gratefully acknowledged.

References


