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NITRIC OXIDE AND NITRIC OXIDE SYNTHASE mRNA INDUCTION IN MOUSE ISLET CELLS BY INTERFERON-γ PLUS TUMOR NECROSIS FACTOR-α

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It has been shown that nitric oxide (NO) is involved in islet cell damage induced by interleukin-1 (IL-1). Here we show that interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) synergistically induced NO production and inducible NO synthase (iNOS) mRNA expression in mouse islet cells. Cycloheximide (CXH) did not prevent the iNOS mRNA expressions. The combination of IFN-γ and TNF-α, which is highly cytotoxic to mouse islet cells, failed to destruct islet cells in the absence of L-arginine or in the presence of N^G-monomethyl-L-arginine (NMMA). These observations suggest that NO is a primary effector in islet cell damage caused by IFN-γ plus TNF-α.

It has been regarded that free radical-induced DNA fragmentation may be a common mechanism by which islet cells were damaged in vitro and in vivo (1-3). Several lines of evidence suggest that NO is involved in islet cell damage associated with insulitis in Type 1 (insulin-dependent) diabetes. NO was recently identified as the primary toxic effector molecule in the lysis of islet cells by inflammatory macrophages (4,5). It has been shown that the deleterious effect of IL-1 on β-cell function (6-11) is associated with the generation of NO by islet cells (12-14).

NO is formed by the mixed functional oxidation of L-arginine to NO and L-citruline by NOS. At least two distinct forms of NOS have been described. Neuron-type NOS (15) existing in the brain, vascular endothelium, lung, adrenal gland, and platelets is a constitutive enzyme which requires calcium as a cofactor. Macrophage-type iNOS exists in macrophages,

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Abbreviations: IL-1, interleukin-1; IFN-γ, interferon-γ; TNF-α, tumor necrosis factor-α; NO, nitric oxide; NOS, nitric oxide synthase; iNOS, inducible nitric oxide synthase; CHX, cycloheximide; NMMA, N^G-monomethyl-L-arginine; RT-PCR, reverse transcribed polymerase chain reaction.

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neutrophils, and Kupffer cells. Recent cloning and characterization of mouse iNOS (16) enabled us to analyse iNOS mRNA in islet cells by reverse transcribed polymerase chain reaction (RT-PCR).

The synergistic interaction between IFN-γ and TNF-α results in the lysis of islet cells (17-19). Although it has been suggested that free radicals are involved in the synergistic cytotoxicity (20-24), the precise mechanism has not been elucidated. In this study we found that not only IL-1 but the combination of IFN-γ plus TNF-α induced iNOS mRNA expression by mouse islet cells.

MATERIALS AND METHODS

Islet cells and cytokines.

Islets were isolated by a collagenase digestion technique from the pancreases of B10.GD mice and purified by two-step handpicking to eliminate exocrine tissues. Islets were incubated with 1 mmol/l glycoletherdiaminetetra acetic acid, and dispersed to single cells with 2 mg/ml collagenase type IV (Worthington, NJ) and 100 U/ml DNase (Sigma, MO). After adhesive non-endocrine cells were removed, cells were cultured in 10% fetal bovine serum supplemented RPMI-1640 medium without phenol red (Gibco, NY). Recombinant mouse IL-1α (8×10^6 U/mg) and recombinant mouse TNF-α (4×10^7 U/mg) were provided by Genzyme (MA). Recombinant mouse IFN-γ (6×10^6 U/mg) was provided by Shionogi (Osaka, Japan).

Measurement of nitrite.

After 24-h primary culture, dispersed islet cells (2×10^4 cells/well) were refed with 100 μl of fresh medium containing cytokines and incubated for 24 h. Medium samples were centrifuged at 14,000 rpm at 4℃ for 10 min. Nitrite was analyzed by the method of Green et al. (25) with minor modifications. To the supernatants (45 μl) were added 45 μl of saline and 10 μl reagent containing 0.5% naphthylethylenediamine dihydrochloride (Wako, Osaka, Japan), 5% sulfanilamide (Wako), and 25% H_3PO_4 (Wako). After 15-min incubation at 60℃, the absorbance at 546 nm was measured by a spectrophotometer (Simadzu, Kyoto, Japan). Standard samples of sodium nitrite were prepared in the mixed solution of saline and the complete medium. The detection limit of nitrite was approximately 150 nmol/l. In studies to assess the effect of CHX on nitrite production, medium was changed to that containing 10 μmol/l CHX (Wako) 10 min before the addition of cytokines.

RT-PCR of iNOS mRNA.

Dispersed islet cells were cultured at a density of 5×10^4/well for 24 h, followed by a 12-h incubation in 500 μl/well of complete medium containing cytokines. RNA was extracted by the single-step method (26) using RNAzol B (Biotecx, Texas), and 100 ng of RNA was converted to cDNA with 10 μg/ml of oligo$(dT)_{12\text{-}18}$ (Pharmacia LKB, Tokyo, Japan), 1 mmol/l of dNTPs (Takara, Kyoto, Japan), 10 mmol/l dithiothreitol (Gibco), 3 mmol/l $MgCl_2$, and 200 U of Moloney murine leukemia virus reverse transcriptase (Bethesda Res, MA) at 37℃ for 60 min and at 42℃ for 30 min.

The amplification of iNOS cDNA sequence was carried out by PCR using a temperature control system (Astec, Fukuoka, Japan) in 50 mmol/l Tris-HCl buffer pH 9 containing 20 mmol/l of NH_2SO_4, 0.7 mmol/l of $MgCl_2$, 200 μmol/l of dNTPs and 50 U/ml of Tub DNA polymerase (Amersham, UK) with following oligonucleotide primers: 5'-CCACCTTGTTCAG-CTACGCC-3' (1655-1674) and 5'-GGACATCAAAGGTCTCACAG-3' (2022-2041). cDNA sequence of β-actin was simultaneously amplified as control using primers 5'-ATCCGTAAA-

GACCTCTATGC-3' (945-964) and 5'-AACGCAGCTCAGTAACAGTC-3' (1212-1231). A thermal cycle was 30 sec at 95 ℃, 30 sec at 55 ℃ and 30 sec at 72 ℃. After 5-cycle amplification with iNOS primers alone, β-actin primers were added to reaction mixtures followed by 25-cycle amiplification. PCR products were analyzed on 3% agarose gels (Nippongene, Toyama, Japan).

Cytotoxicity assay.

Islet cells were inoculated at 4×10^4 cells/well in 96-well culture trays. After 24-h culture, cells were refed with 100 μl/well of complete medium containing 200 U/ml IFN-γ and 200 U/ml TNF-α with or without NMMA. In a study to assess the effect of L-arginine on cytokine-induced islet cell lysis, L-arginine-free RPMI-1640 medium (Gibco) was used. After 5-day exposure, medium samples were obtained for nitrite assay, and both nonadhesive cells and adhesive cells resuspended by trypsin/EDTA were harvested. Viable and nonviable cells were counted in hemocytometers using the trypan-blue dye exclusion method.

Statistics.

Results are expressed as means and SD. Statistical analyses of significance were performed using unpaired Student's t test.

RESULTS

Mouse IL-1α of 10-100 U/ml augmented nitrite production by islet cells during a 24-h incubation period (Fig. 1), whereas neither IFN-γ nor TNF-α of 50-200 U/ml enhanced nitrite generation. However, exposure to the combination of 200 U/ml IFN-γ and 200 U/ml TNF-α resulted in marked nitrite production by islet cells. It has been reported that CHX inhibits IL-1-induced NO generation by rat islet cells. In this study CHX proved to suppress the effect of the combination of IFN-γ plus TNF-α as well as that of IL-1α. Figure 2 shows progressively stimulated nitrite production by islet cells induced by IFN-γ and TNF-α after an initial lag period of ~6 h.

To determine whether IL-1α induces iNOS mRNA expression in mouse normal islet cells, and whether the synergy between IFN-γ and TNF-α is mediated through the induction of iNOS,

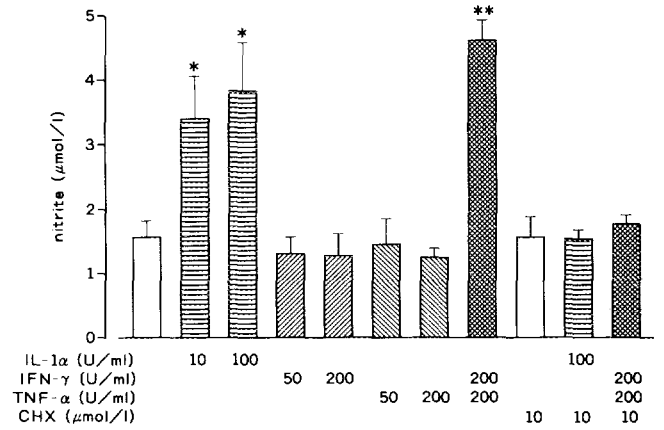

Figure 1. Nitrite production by mouse islet cells. Dispersed islet cells (2×10^4 cells/100 μl) were exposed to IL-1α, IFN-γ and/or TNF-α in the presence or absence of CHX for 24 h. Means and SD (n=4). *p<0.01, **p<0.001.

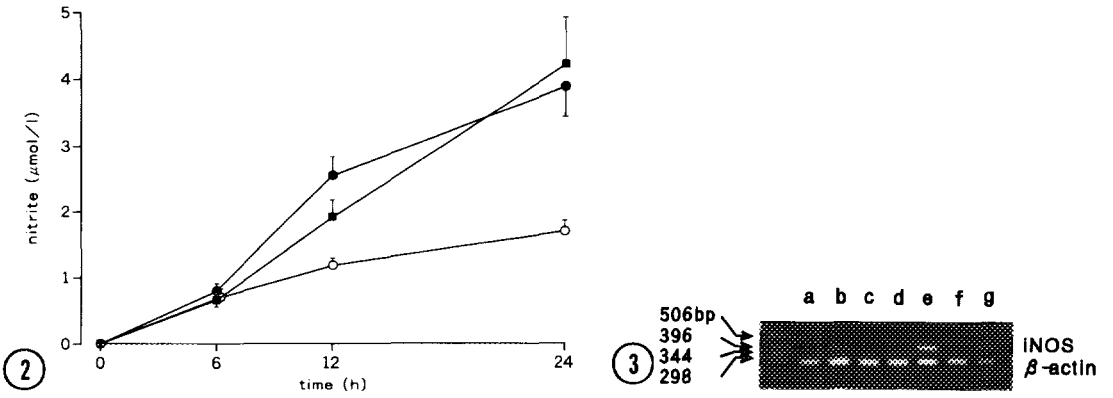

Figure 2. Medium nitrite levels of islet cell cultures. Mouse islet cells (2×10^4 cells/well) were incubated in 100 μl culture medium supplemented with 100 U/ml IL-1α (●) or 200 U/ml IFN-γ plus 200 U/ml TNF-α (■), or without cytokines (○).

Figure 3. Cytokine-induced expression of iNOS mRNA by mouse islet cells. Ethidium bromide-stained agarose gel of 387 bp PCR products of iNOS cDNA and 287 bp sequences of β-actin is shown. a, no cytokines; b, 100 U/ml IL-1α; c, 200 U/ml IFN-γ; d, 200 U/ml TNF-α; e, 200 U/ml IFN-γ plus 200 U/ml TNF-α; f, 100 U/ml IL-1α and 10 μmol/l CHX; g, 200 U/ml IFN-γ plus 200 U/ml TNF-α and 10 μmol/l CHX.

expression of its mRNA in islet cells was assessed by RT-PCR. As is demonstrated in Figure 3, iNOS mRNA which was not constitutively expressed by islet cells, was induced by IL-1α. Although neither IFN-γ nor TNF-α alone did not induce detectable iNOS mRNA expression, synergistic interaction between IFN-γ and TNF-α resulted in the expression of this mRNA. The iNOS mRNA expressions were not inhibited by CHX, indicating that de novo protein synthesis is not required to the iNOS mRNA induction by IL-1α or IFN-γ plus TNF-α.

Since islet cells are vulnerable to NO, synergistic cytotoxic action of IFN-γ and TNF-α may be attributable to augmented NO production by islet cells. Five-day incubation of islet cells with IFN-γ and TNF-α resulted in cell destruction (Table 1). Marked accumulation of nitrite was shown in the culture medium supplemented with the cytokines. However, in medium containing no L-arginine, the cytokines failed to destruct islet cells. NMMA, an NOS inhibitor, lowered nitrite levels and protected islet cells from the cytotoxic action of IFN-γ plus TNF-α.

DISCUSSION

Several authors including ourselves have reported that free radical scavengers protected islet cells against the cytotoxic action of IFN-γ and TNF-α (20-24). However, the radical(s)

Table 1. Effects of NMMA and L-arginine on nitrite production and islet cell destruction induced by 5-day exposure to the combination of IFN-γ plus TNF-α

	nitrite (μmol/l)	viability (%)
blank	4.4 ± 0.8	98.3 ± 0.5
IFN-γ 200 U/ml, TNF-α 200 U/ml	41.4 ± 2.0	39.6 ± 11.5
minus L-arginine	6.5 ± 1.1 *	97.4 ± 1.8 *
plus NMMA 500 μmol/l	10.3 ± 4.0 *	92.7 ± 3.8 *

mean ± SD, n=4, *p<0.001 vs IFN-γ plus TNF-α.

responsible for islet cell damage has not been identified. We demonstrated in this study that exposure of islet cells to the combination of IFN-γ and TNF-α resulted in the elevation of nitrite levels in culture medium. This observation indicates that the cytokines induced NO production by islet cells, since NO is markedly unstable and rapidly oxidized to nitrite in aerobic condition. The nitrite induction required the synergistic interaction between IFN-γ and TNF-α; neither IFN-γ nor TNF-α by itself augmented nitrite production. Basal production of NO by islet cells is probably catalyzed by neuron-type NOS, although its physiological role remained to be clarified.

It has been reported that IL-1-induced NO production results in the inactivation of an iron-containing Krebs cycle enzyme aconitase in rat islet cells (9). Furthermore, exposure of islet cells to NO leads to DNA strand breaks (27). Okamoto et al. (1-3) has shown that both streptozotocin and alloxan generate reactive intermediates resulting in DNA strand breaks and poly(ADP-ribose) synthesis, which consumes NAD as a substrate to reduce the NAD contents and inhibit proinsulin synthesis. He has suggested that it may be a common pathway to damage islet cells. Previously we have reported that the combination of IFN-γ and TNF-α reduces the NAD content of islet cells (23). Here we showed that NMMA inhibited nitrite production and cell destruction induced by the cytokines. The cytokines did not damage islet cells in the absence of L-arginine. These observations indicate that NO plays an effector role in the islet cell cytotoxic action of IFN-γ plus TNF-α probably through DNA fragmentation.

IL-1 has been regarded to stimulate NO production through NOS gene transcription and NOS protein synthesis, since actinomycin-D and CHX inhibited the IL-1-induced NO generation (11-13). IL-1β-induced iNOS expression was reported in hamster insulinoma cells by the Northern blot analysis (28). Here we showed by amplifying an iNOS mRNA-specific sequence that IL-1α actually induced iNOS mRNA expression by normal islet cells. Since exocrine cells and intraislet adhesive cells had been removed from the cell preparations, it can be assumed that nitrite was mainly produced by endocrine cells. The potential of normal β-cells to produced NO was shown by Corbett et al. (13) using purified β-cells and IL-1β. However, the possibility remains that a small number of intraislet nonendocrine cells may partially participate in cytokine-induced iNOS mRNA expression.

IL-1 directly induces the synthesis of many cellular proteins in various cells. Because IL-1 provokes de novo synthesis of TNF-α by islet cells (29,30), endogenous TNF-α might be involved in the IL-1-induced NO generation. However, RT-PCR studies showed that CHX did not significantly suppress IL-1- or the cytokine combination-induced iNOS gene transcription. IL-1 and the combination of IFN-γ plus TNF-α may be able to induce independently iNOS mRNA expression.

Thus, not only IL-1 but IFN-γ plus TNF-α induced NO generation by mouse islet cells through augmented iNOS gene transcription. The cytokines may act synergistically with the NO derived from macrophages resulting in the impairment of insulin secretion and cell destruction.

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