Transgenic Mice Overexpressing Type 2 Nitric-oxide Synthase in Pancreatic β Cells Develop Insulin-dependent Diabetes without Insulitis*

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We generated transgenic mice carrying the mouse type 2 nitric-oxide synthase (NOS2) cDNA under the control of the insulin promoter. Western and immunohistochemical analyses revealed that NOS2 was expressed abundantly in transgenic islets but not in control islets. When islets were isolated and cultured, high levels of nitrite were released from the transgenic islets. In transgenic mice, the β cell mass was markedly reduced without the infiltration of macrophages or lymphocytes, and extensive DNA strand breaks were detected in the islets by in situ nick translation. All the transgenic mice developed hypoinsulinemic diabetes by 4 weeks of age, and treatment with an inhibitor of NOS2, aminoguanidine (200 mg/kg body weight every 12 h), prevented or delayed the development of diabetes. The present study shows that the production of nitric oxide by β cell NOS2 plays an essential role in the β cell degeneration.

Insulin-dependent diabetes mellitus (IDDM) is caused by the degeneration of insulin-producing β cells in pancreatic islets (1–4). Nitric oxide (NO), first identified as a physiological signaling molecule, has been shown to be a cytotoxic effector molecule when generated in high concentrations by type 2 NO synthase (NOS2) (5). In the process of IDDM, activated macrophages produce NO, which is thought to be cytotoxic to β cells, and NO, which is produced by β cell NOS2 induced by macrophage-derived cytokines such as interleukin-1β (IL-1β), is also thought to be involved in β cell degeneration (6). Although many in vitro studies (7–13) suggest that NO produced by cytokine-induced NOS2 can cause the degeneration of β cells, no in vivo study has clearly demonstrated the pathological significance of NO produced within β cells in the development of IDDM, because an infiltration of macrophages in islets always occurred in animal models of IDDM (6, 14). In this study, we produced transgenic mice expressing NOS2 constitutively in pancreatic β cells and found that the transgenic mice developed severe IDDM without macrophage or lymphocyte infiltration in and around islets.

EXPERIMENTAL PROCEDURES

Construction of Rat Insulin II Promoter/Mouse NOS2 Hybrid Gene—Islets were isolated from ICR mice by the collagenase digestion method (15) and cultured for 12 h in the presence of 150 units/ml IL-1β (Sigma). A mouse NOS2 cDNA was cloned by polymerase chain reaction (PCR) of reverse-transcribed RNA from IL-1β-stimulated islets. Primers used in the PCR reaction were 5′-TTCCGGGAGCAGAAGTGCAAAGTCTCA-3′ and 5′-AAAGATCTGGGCTCTAGAGCTGTCGGTTT-3′; these sequences correspond to the nucleotides −25 to −1 and 3418 to 3444 of mouse NOS2 cDNA (16) and contain Xmal and BglII sites (underlined sequences), respectively. The cloned cDNA sequence was determined and was found to be exactly the same as the reported NOS2 sequence (16). To express NOS2 in β cells, the 0.7-kbp BamHI-Xmal fragment of the rat insulin II promoter (17), the 1.6-kbp BglII-EcoRI fragment of the SV40 intron/polyadenylation signal (18) were ligated at the Xmal and BglII sites in the correct orientation. The resultant hybrid gene (5.8 kbp) separated from the pBlueScript SK− (−) (Stratagene) by KpnI and NotI was used for microinjection.

Generation of Transgenic Mice—To generate transgenic mice, a DNA solution (2 μg/ml) was microinjected into the male pronuclei of fertilized eggs from BDF1 females as described (17, 19). Identification of transgenic mice was performed by PCR on genomic DNA. In the present study, the two diabetic transgenic lines, 31 and 40, were established, maintained on CD-1 mouse background, and analyzed. Because the diabetes occurred in two independent lines of transgenic mice, the pathology was assumed to have resulted from the transgene expression rather than from the positional or insertional effects of the transgene.

Western Blot and Immunohistochemical Analyses—Pancreatic islets were isolated from 4–8-week-old mice by collagenase digestion (15) and homogenized (19). Protein concentrations were determined using BCA Protein Assay Reagent (Pierce). Western blot and immunohistochemical analyses of NOS2 were carried out as described (17, 20) using the diluted (1:2000) rabbit anti-mouse NOS2 antibody (Wako, Osaka, Japan) (21). Insulin staining was carried out as described (17) using anti-porcine insulin antibody (DAKO, Carpinteria, CA).

Measurement of Nitrite Release from Isolated Islets—50–200 islets, isolated from transgenic and nontransgenic mice, were incubated in 150 μl of RPMI 1640 medium without phenol red (Sigma) containing 10% fetal calf serum (JRH Biosciences, Lenexa, KS) and 11.1 mM glucose. Pancreatic extracts were obtained from the entire pancreas by acidethanol extraction (23). The insulin levels in the serum and pancreatic extracts were determined by using an insulin radioimmunoassay kit (Amersham Corp.) and rat insulin standards.

Measurement of Glucose and Insulin—Blood and urinary glucose levels were determined by using Advantage equipment (Boehringer Manheim) and Tes-tape assay (Lilly, Indianapolis, IN), respectively. Pancreatic extracts were obtained from the entire pancreas by acidethanol extraction (23). The insulin levels in the serum and pancreatic extracts were determined by using an insulin radioimmunoassay kit (Amersham Corp.) and rat insulin standards.

Analyses of DNA Strand Breaks by Nick Translation—Pancreata...
RESULTS AND DISCUSSION

Western blot analysis using an NOS2-specific antibody showed that the transgenic mice (lines 31 and 40), but not the nontransgenic control mice, expressed NOS2 in the pancreatic islets (Fig. 1A). The NOS2 expression was not detected in other tissues such as brain, liver, kidney, and small intestine of the transgenic mice (Fig. 1B). NOS2 mRNA was detected only in the transgenic islets by Northern blot analysis (data not shown). In immunohistochemistry, islets of transgenic mice were stained for NOS2 (Fig. 2, B and C). The NOS2 expression in transgenic islets was observed from 1 week of age (data not shown). The proportion of insulin-producing cell mass to total pancreatic cell mass was markedly reduced in the transgenic mice (Fig. 2, D–G). On the other hand, islets of the control mice showed no immunoreactivity for NOS2, and the pancreatic exocrine cells showed no detectable staining for NOS2 in the transgenic and nontransgenic mice (Fig. 2, A–C).

We next incubated islets from transgenic and control mice and measured the nitrite content in the incubation medium, which is indicative of NO release (16). As shown in Fig. 3, high levels of nitrite were detected in a time-dependent manner in the medium containing transgenic islets, whereas the nitrite content in the control islet medium was under the limit of detection. The amount of nitrite in the medium of transgenic line 31 was almost equivalent to that of the medium containing IL-1β-stimulated control islets.

In the transgenic lines 31 and 40, the mice developed hyperglycemia from 1 week of age (Fig. 4A) and exhibited profound polydipsia/polyuria by 4 weeks of age. The blood glucose levels of both lines of transgenic mice were over 400 mg/dl, and the urine tested strongly positive (250–500 mg/dl) for glucose. The nontransgenic control mice had normal glucose levels (less than 200 mg/dl) and never showed glycosuria. The serum insulin levels in the transgenic lines 31 (0.69 ± 0.07 ng/ml, n = 7) and 40 (0.53 ± 0.07 ng/ml, n = 7) were significantly (p < 0.05) lower than those in the control mice (3.68 ± 1.10 ng/ml, n = 7). Urine from both lines of transgenic mice often tested positive for ketones; the presence of ketonuria indicated the severity of the diabetes. We followed the diabetes development every week from the first week of age by monitoring the blood glucose levels and pancreatic insulin contents. As shown in Fig.

![Western blot analysis of homogenates from islet (A) and various tissues (B).](image)

![Immunohistochemical detection of NOS2 in mouse pancreas (A–C), insulin staining of representative sections of pancreas (D–F), and islet cell mass of transgenic mice (G)](image)
4, the transgenic mice developed diabetes from as early as 1 week of age. The diabetes was clearly insulin-dependent, because the blood glucose level of diabetic mice injected with 4 units of exogenous insulin (Humulin U, Lilly) was reduced to below 200 mg/dl (data not shown). These results indicated that the diabetes observed in the transgenic mice was characteristic of IDDM (type 1 diabetes, ketosis prone) (27). At 1 week of age, NOS2 was immunohistochemically detected in the islets of the transgenic mice, suggesting that NOS2 expression was correlated with the development of diabetes. There was no evidence of transient insulitis such as infiltration of macrophages or lymphocytes at any time from the first to the eighth week of age (Fig. 2).

It has been reported that aminoguanidine, an inhibitor of NOS2 (28), was effective in reducing NO\textsubscript{z} produced by IL-1\textbeta\textsubscript{-}treated islets \textit{in vitro} (29). To demonstrate that the diabetes in the transgenic mice was dependent on NO\textsubscript{z} production, we undertook a prophylactic intervention with aminoguanidine. The elevation of blood glucose levels as well as the reduction of pancreatic insulin contents in the transgenic mice were prevented (line 31) or delayed (line 40) by aminoguanidine treatment (Fig. 4). In control mice, the intraperitoneal administration of aminoguanidine did not affect the body weight, blood glucose levels, or serum insulin levels during the 8 weeks. Furthermore, the reduction of the insulin-producing cell mass was also prevented or improved by aminoguanidine (Fig. 2G), indicating that the transgenic model represents the effect of NO\textsubscript{z} produced by NOS2 in β cells. Immunohistochemical staining of NOS2 showed that the transgenic mice treated with aminoguanidine did express NOS2 (data not shown), indicating that the effects of aminoguanidine were not due to the loss of expression of specific transgenes under the control of the insu-
lin promoter. It has been reported that aminoguanidine treatment has no effect on the appearance of insulitis or the incidence of diabetes in animal models of immune-mediated IDDM (14, 30, 31). It is reasonable to assume that not only NO but also other β cytotoxic factors such as hydroxyl radicals and direct cytotoxic actions by cytotoxic T lymphocytes can be involved in the development of the immune-mediated model (3, 4).

Because high concentrations of exogenous NO have been shown to cause islet DNA strand breaks in vitro (8, 9), we examined DNA strand breaks in transgenic and control islets. DNA was labeled by nick translation and analyzed by gel electrophoresis. As shown in Fig. 5A, [32P]dCTP was incorporated into transgenic islet DNA but not into control islet DNA. The estimated incorporation of [32P]dCTP into transgenic islet DNA (line 31, 27.2 ± 0.98 cpm/pg DNA; line 40, 45.1 ± 1.58 cpm/pg DNA) was much higher than that into control DNA (0.66 ± 0.06 cpm/pg DNA). We further confirmed the presence of DNA strand breaks by in situ nick translation. Transgenic islet cells showed extensive DNA strand breaks (Fig. 5, C and D), but nontransgenic islet cells did not (Fig. 5B). We and others have reported that when NO is produced in β cells upon cytokine stimulation (4, 6–12), extensive DNA strand breaks can occur to initiate a “suicidal” response; once β cell DNA strand breaks occur, nuclear poly(ADP-ribose) synthetase is activated, causing the depletion of intracellular NAD+ (3). The depletion of NAD+ severely impairs β cell functions and ultimately evokes β cell death (3, 4, 15, 32–35).

We have already proposed that although IDDM can be caused by many different agents such as immunologic abnormalities, inflammatory tissue damage, and β cytotoxic chemical substances, the final pathway for the toxic agents is the same (3, 4, 12, 15, 32–35). This pathway involves DNA damage by free radicals such as NO and hydroxyl radicals, poly(ADP-ribose) synthetase activation, and NAD+ depletion. Therefore, IDDM is theoretically preventable by suppressing immune reactions, scavenging free radicals, and inhibiting poly(ADP-ribose) synthetase by its inhibitors. The mechanism of β cell death and its prevention was confirmed by using poly(ADP-ribose) synthetase gene disrupted mice (13). In the present study, we showed that NOS2 transgenic mice developed IDDM with β cell DNA damages by NO produced in the cells. Moreover, it was recently reported that NO-mediated poly(ADP-ribose) synthetase activation plays an essential role in ischemic neuronal cell death (36). It is thus reasonable to assume that not only β cell death in IDDM but also many other cell deaths such as ischemic brain injury can be explained by the pathway described above.

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