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Spin trapping agent phenyl *N-tert*-butylnitrone protects against the onset of drug-induced insulin-dependent diabetes mellitus

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Abstract Insulin-dependent diabetes mellitus is an autoimmune disease believed to be caused by an inflammatory process in the pancreas leading to selective destruction of the β-cells. Cytokines and nitric oxide (NO) have been shown to be involved in this destruction. Phenyl *N-tert*-butylnitrone (PBN) has demonstrated protective effects against several pathological conditions including ischemia-reperfusion injury and endotoxin-induced shock. We report here that PBN co-administration can prevent the onset of the STZ-induced diabetes in mice. PBN co-treatment inhibited the streptozotocin (STZ)-induced hyperglycemia, the elevation in the level of glycated hemoglobin and weight loss in the treated mice. Histological observations indicated destruction of β-cells in the STZ-treated animals and its prevention by PBN co-treatment. EPR spin trapping experiments in the pancreas indicated the in vivo formation of NO in STZ-treated animals and its attenuation by PBN treatment.

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Key words: Diabetes; Streptozotocin; Phenyl *N-tert*-butylnitrone; Nitric oxide; Electron paramagnetic resonance; Spin trap

1. Introduction

Insulin-dependent diabetes mellitus (IDDM) is an autoimmune disease characterized by a local inflammatory reaction in and around the islets of Langerhans in the pancreas. This inflammatory response, also known as insulitis, is caused by infiltration of monocytes, macrophages, and T-lymphocytes with the ultimate consequence of selective destruction of the insulin secreting β-cells in the pancreas [1]. Several lines of evidence point to the involvement of the pro-inflammatory cytokines in the pathogenesis of IDDM. Interleukin-1 (IL-1), tumor necrosis factor-α, interferon-γ (IFN-γ), and particularly the combination of these cytokines have been shown to impair β-cell function and to be cytotoxic to β-cells in vitro [1–4]. Non-obese diabetic (NOD) mice given antibodies to IFN-γ and IL-6 showed reduced severity of insulitis and a lower incidence of autoimmune diabetes [5]. A soluble IL-1 receptor was found to protect against diabetes in NOD mice [6] and an IL-1 receptor antagonist was shown to prevent the diabetogenic process elicited by the drug streptozotocin (STZ) [7]. The free radical nitric oxide has also been implicated in the development of IDDM. STZ-induced islet destruction and hyperglycemia in mice was reduced by the nitric oxide synthase (NOS) inhibitor L-N^G-monomethylarginine [8]. Islets isolated from NOD mice were found to produce nitrite (an oxidation product of NO) in vitro [9]. Selective inhibition of the inducible isoform of NOS (iNOS) by aminoguanidine was shown to prevent the IL-1-mediated hyperglycemia and hypoinsulinemia [10].

The compound phenyl *N-tert*-butylnitrone (PBN), which was originally developed as a free radical trapping agent [11], has a variety of pharmacological effects. These effects include the protective action against the central nervous system (CNS) damage brought about by ischemia-reperfusion [12,13], the reduction of the mortality caused by endotoxin-induced septic shock [14,15], and the amelioration of the CNS damage associated with the HIV envelope protein gp120 administration in rat neonate model [16]. Recently, Miyajima and Kotake [14,15] have demonstrated that the protective effect of PBN in septic shock is likely due to the ability of this compound to inhibit the induction of iNOS. In this study we have investigated whether PBN co-administration can prevent STZ-induced IDDM in a mouse model. In vivo generation of NO in the pancreas as a result of STZ administration and the possible effect of PBN on this phenomenon have also been studied using electron paramagnetic resonance (EPR) spectroscopy.

2. Materials and methods

Streptozotocin was obtained from Sigma Chemical Company (St. Louis, MO). PBN was obtained from OMRF Spin Trap Source (Oklahoma City, OK). *N*-(dithiocarboxy) sarcosine, in an iron complex form was a gift from Dr. T. Yoshimura, Institute for Life Support Technology, Yamagata, Japan. Other chemicals were obtained from Sigma Chemical Co.

Balb/c mice (male, 6–8 weeks) from Charles-River (Indianapolis, IN) were randomly divided into four groups (five mice per group) and were given intraperitoneal (i.p.) injections for 5 consecutive days of the following reagents: STZ, 40 mg/kg (STZ group); STZ, 40 mg/kg + PBN, 150 mg/kg (STZ+PBN group); PBN, 150 mg/kg (PBN group); and phosphate buffer saline (PBS group). STZ was dissolved in citrate buffer (pH 4.5) and was administered within 10 min of dissolving. Injection volume for each treatment was 0.2 ml. Animals receiving PBN co-treatment were injected with this compound 15 min prior to STZ treatment. This group of animals received one extra injection of PBN only (150 mg/kg) at day 6. At different time intervals after the treatments, tail tip blood was drawn and used for the measurements of blood glucose level or glycated hemoglobin. Blood glucose level was determined by the glucose oxidase kit obtained from Sigma Chemical Co. Glycated hemoglobin (Hb A_1) was measured using a kit from Sigma.

For histological studies, anaesthetized mice were perfused with 4% paraformaldehyde and pancreata were removed and post-fixed for an

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Abbreviations: STZ, streptozotocin; NO, nitric oxide; PBN, phenyl *N-tert*-butylnitrone; IL-1, interleukin-1; iNOS, inducible nitric oxide synthase; EPR, electron paramagnetic resonance; Fe-DTCS, iron complex of *N*-(dithiocarboxy) sarcosine

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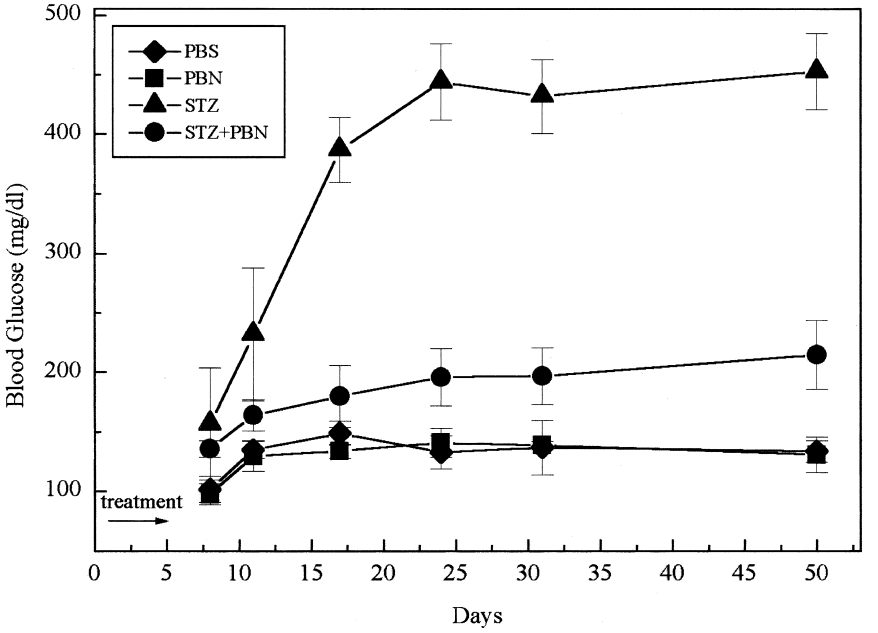

Fig. 1. Effect of PBN co-administration on STZ-induced hyperglycemia. Mice were treated with PBS (♦); PBN, 150 mg/kg (■); STZ, 40 mg/kg (▲); or STZ, 40 mg/kg + PBN, 150 mg/kg (●) for 5 consecutive days. Values are reported as mean ± SD.

additional day. The paraffin sections were stained with Gomori's aldehyde fuschin for the identification of pancreatic β-cells [17].

For in vivo trapping of NO in the pancreas of the STZ-treated animals, mice were treated with a single dose of STZ (120 mg/kg, i.p.). Some animals received PBN pretreatment (300 mg/kg) 30 min prior to STZ injection. Five and one-half hours after STZ administration, animals received an i.p. injection of the newly developed NO spin trap, iron complex of *N*-(dithiocarboxy) sarcosine (Fe-DTCS, 80 mg/kg) [18]. Mice were killed 30 min after receiving the spin trap, the pancreata were immediately removed and placed on the EPR tissue cell (Wilmad, Buena, NJ) and the EPR spectra were recorded using a Bruker 300 electron spin resonance spectrometer at room temperature. EPR parameters were as follows: receiver gain, 5.00×10^5; modulation amplitude, 2.0 gauss; modulation frequency, 100 kHz. Statistical analyses were performed by 1-way analysis of variance (ANOVA) followed by Students' *t*-test.

3. Results

In order to determine whether PBN co-treatment would protect the animals against the diabetogenic action of STZ, the effect of such treatment was determined on several indices of diabetes, namely, blood glucose level, the level of glycated hemoglobin, the weights of the animals, and the pancreata

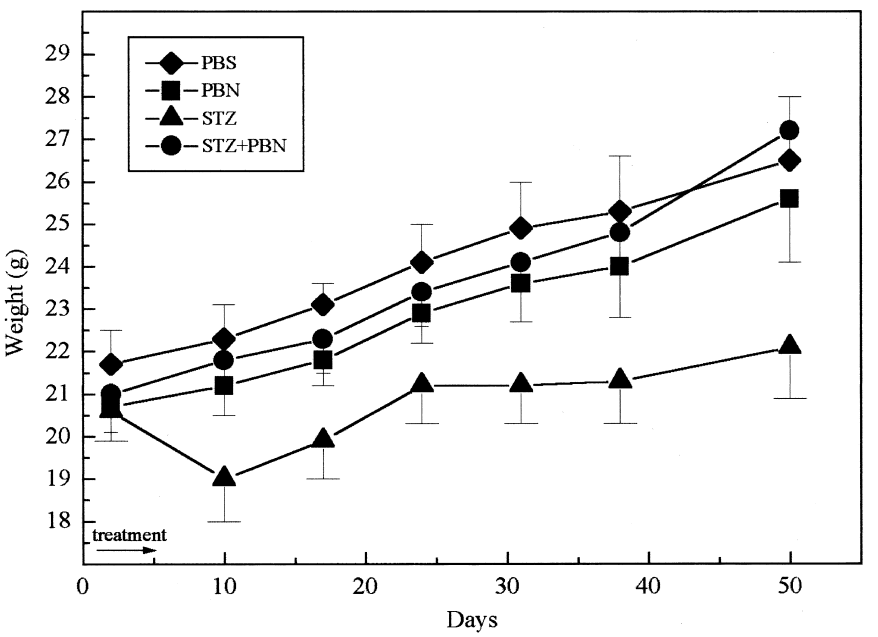

Fig. 2. Effect of PBN co-administration on STZ-induced weight loss. Mice were treated with PBS (♦); PBN, 150 mg/kg (■); STZ, 40 mg/kg (▲); or STZ, 40 mg/kg + PBN, 150 mg/kg (●) for 5 consecutive days. Values are reported as mean ± SD. The positive or negative half of each error bar has been demonstrated.

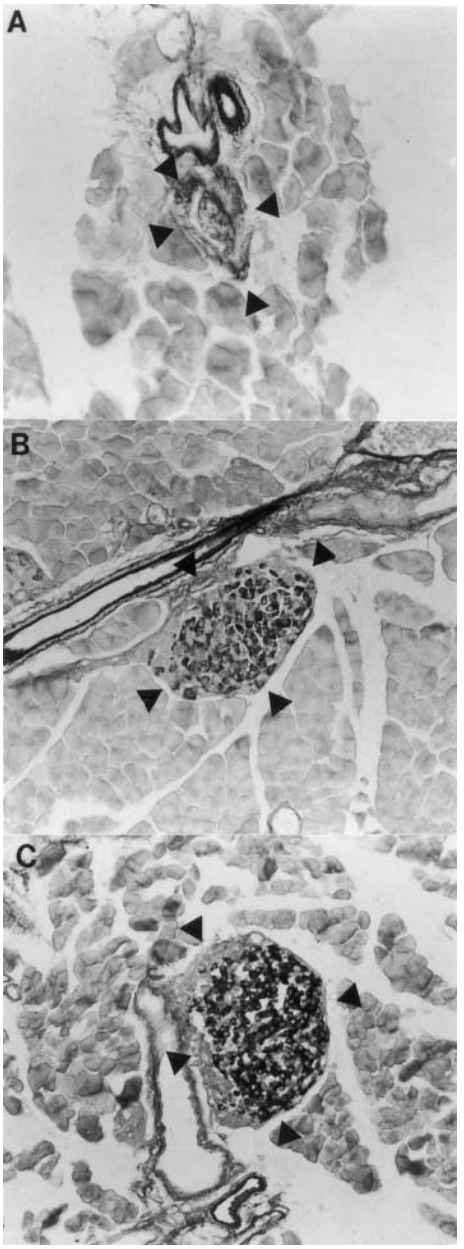

Fig. 3. Microscopic images of mouse pancreatic tissue. A: In STZ-treated mice pancreatic islets, and the β cells (dark cells) contained within those islets are virtually absent in pancreatic tissue. B: In animals treated with a combination of STZ+PBN, pancreatic β cells (dark cells) are found in morphologically normal appearing islet tissue. Arrowheads indicate pancreatic islets. C: In PBN-treated animals, pancreatic β cells remain intact. Magnification: 172×.

histology. Mice treated with STZ demonstrated a progressive rise in the blood glucose concentration which reached a plateau at ca. 450 mg/dl by 3 weeks post-treatment. The blood glucose level of the animals which received PBN co-treatment, however, stayed near normal throughout the experiment (Fig. 1). The differences between the two groups (STZ vs. STZ+PBN) were statistically significant ($P < 0.05$ at day 11, and $P < 10^{-5}$ at day 17 and thereafter). Mice treated with either PBS or PBN displayed blood glucose levels within the normal range throughout the experiment. No statistically significant differences were observed between the blood glucose levels of these two groups.

Table 1
Effect of PBN co-administration on STZ-induced increase in % glycated hemoglobin

Treatment	% Glycated hemoglobin[a]	
	Day 30	Day 50
PBS	4.24 ± 0.45	4.03 ± 0.22
PBN	4.62 ± 0.22	3.97 ± 0.16
STZ	9.16 ± 0.64	9.10 ± 0.76
STZ+PBN	4.79 ± 0.46**	4.63 ± 0.28**

[a]Values are reported as the mean ± standard deviation, $n = 5$.**Values are significantly different ($P < 0.01$) from STZ-treated group.

The STZ-treated mice demonstrated increasingly elevated levels of glycated hemoglobin. PBN co-administration maintained the glycated hemoglobin level in the treated animals close to the control values. The level of glycated hemoglobin in the STZ+PBN group was not significantly different from the PBS group at 30 days following treatment ($P = 0.88$). At 50 days, a small but statistically significant ($P = 0.005$) increase in the glycated hemoglobin was observed in the STZ+PBN versus the PBS group. As expected, the mice receiving either PBS or PBN (without STZ) had low levels of glycated hemoglobin (Table 1).

The STZ-treated animals demonstrated an initial weight loss followed by a weight gain the rate of which was significantly lower than the control (PBS) group (Fig. 2). PBN, however, prevented the STZ-induced weight loss and the weights of the STZ+PBN group remained very similar to those of the control animals. The differences between the weights of the two groups were found to be statistically significant by day 11 ($P \leq 0.001$). Mice receiving PBN only showed a small but statistically significant decrease in their weights when compared to the PBS-treated mice for up to 20 days after treatment ($P < 0.05$). The reason for such weight loss is yet to be determined.

Histological inspection of pancreatic tissue in STZ-treated mice indicated that the pancreatic islets and β-cells were destroyed (Fig. 3A). In animals treated with a combination of

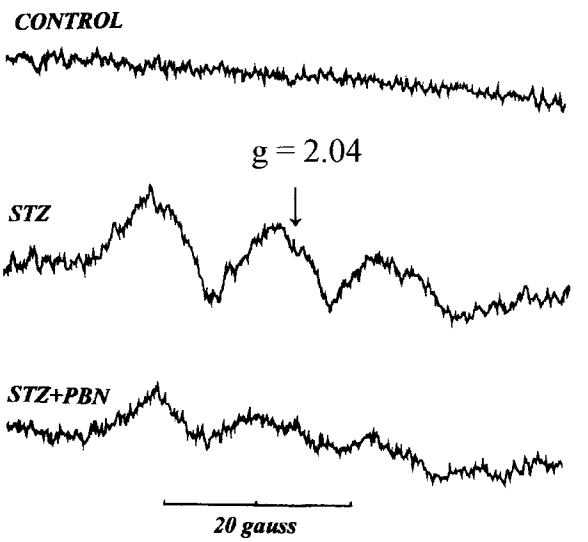

Fig. 4. EPR spectra obtained in the pancreata of the STZ-treated mice and the effect of PBN co-treatment. Mice were treated with PBS (CONTROL); STZ, 120 mg/kg (STZ); and STZ, 120 mg/kg+PBN, 300 mg/kg (STZ+PBN). See Section 2 for experimental details.

PBN+STZ, pancreatic integrity was visually maintained but the number of β-cells within the islets appears to be slightly reduced (Fig. 3B) as compared to PBN control (Fig. 3C). It is clear that the STZ-induced destruction of the islets was prevented in this tissue.

In order to determine whether STZ administration would cause the formation of NO in the pancreas of the treated mice, animals were treated with the newly developed NO spin trap Fe-DTCS following a single high dose of STZ (120 mg/kg) and EPR spectra of the pancreata of the treated animals were recorded. The characteristic triplet signal due to nitrogen with a hyperfine splitting of 13.0 gauss (g=2.04) was evident in these tissues (Fig. 4). PBN co-administration was found to greatly attenuate this signal. No signal was observed in the control (PBS-treated) animals.

4. Discussion

In this study, we have demonstrated that PBN protects against the onset of IDDM in an STZ-induced model of the disease. We also demonstrate STZ-mediated in vivo generation of NO in the pancreas of the treated animals, and the inhibitory effect of PBN in this process.

PBN co-treatment significantly reduced the extent of the STZ-induced hyperglycemia in mice. Animals in the STZ+PBN group displayed blood glucose levels only slightly elevated (Fig. 1), whereas the STZ group showed significantly high blood glucose levels. In agreement with the observed blood glucose concentrations throughout the experiment, the level of glycated hemoglobin (an index of chronic hyperglycemia) was almost doubled in the STZ group (Table 1), however, animals that had been treated with STZ+PBN showed normal level (Table 1).

Because of the lack of ability to uptake glucose in the tissues such as skeletal muscle which require insulin for glucose entry into the cell, insulin-dependent diabetic patients use fat reservoirs and eventually proteins, as energy sources. This results in weight loss among insulin-dependent diabetic subjects in the absence of insulin therapy. Thus weight loss as a function of STZ-treatment and the effect of PBN co-administration was investigated as yet another index of IDDM. The STZ-treated mice weighed significantly less than the control animals at all time points post-treatment (Fig. 2). The mean weight of the STZ+PBN group, however, stayed very close to that of the control group. PBN treatment per se resulted in a small but statistically significant weight loss when compared to the PBS group. In addition, PBN co-treatment preserved the morphological integrity of the pancreatic islet β-cells as indicated by histological studies (Fig. 3A–C).

All these data indicate that PBN can counteract the diabetogenic effect of STZ. STZ treatment has been shown to induce the expression of the cytokine IL-1 since an IL-1 receptor antagonist was found to protect against the STZ-induced hyperglycemia and insulitis [7]. Nitric oxide has also been implicated in the β-cell destruction caused by STZ-treatment. Inhibition of NO generation by NOS inhibitors reduces the hyperglycemia and islet destruction associated with STZ-treatment in mice [8]. IL-1 treatment has been shown to induce NO formation in rat isolated islets as detected by low temperature EPR [19]. Using EPR spin trapping methodology, we have provided direct evidence that STZ administration to mice does indeed result in the formation of NO in the pancreas (Fig. 4). Nitric oxide and its oxidation products are cytotoxic species believed to cause cell damage and death through inactivation of mitochondrial iron-sulfur containing enzymes and direct oxidation (by peroxynitrite) [20,21].

Previously, PBN has been shown to inhibit the induction of iNOS and hence prevent the mortality and morbidity associated with endotoxin treatment in a mouse septic shock model [14,15]. We have recently demonstrated that PBN prevents the NO formation in the CNS of treated rat neonates caused by HIV envelope protein gp120 and counteracts the behavioral retardation associated with such treatment [16]. PBN treatment caused a significant decrease in the level of NO formed in the pancreas as a result of STZ treatment in mice (Fig. 4). Taken together, these observations indicate that PBN may interfere with certain intracellular step(s) in the cytokine cascade initiated via stimulation by extracellular cytokines such as IL-1. A major outcome of the cytokine cascade is overexpression of iNOS and over-production of NO. Such mechanisms may also account for the protective action of PBN against STZ-induced IDDM. It remains to be determined whether the inhibitory effect of PBN on iNOS induction is related to the ability of this nitrone to trap reactive free radicals.

Experiments are underway to determine at what stage(s) of the pathway leading to iNOS induction and NO over-production does PBN intervene. Such information should provide deeper insights into the mechanisms responsible for the development of IDDM. Thus, in conclusion, the data presented here clearly indicate that PBN and possibly chemically related compounds have the potential of becoming strong investigative tools in the area of diabetes research. Furthermore, the data herein presented indicate that PBN and related compounds can potentially serve as therapeutical agents in IDDM by intervening the onset of the disease in individuals with increased risk for developing this disease.

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