EXPERIMENTAL STUDY

Noricandil improves diabetes and rat islet β-cell damage induced by streptozotocin in vivo and in vitro

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Abstract

Objective: N-(2-hydroxyethyl)-nicotinamide nitrate (nicorandil) is a unique anti-anginal agent, reported to act as both an ATP-sensitive K⁺ channel opener (PCO) and a nitric oxide donor. It also has an anti-oxidant action. We examined the effects of nicorandil on streptozotocin (STZ)-induced islet β-cell damage both in vivo and in vitro.

Design and methods: STZ-induced diabetic Brown Norway rats (STZ-DM) were fed with nicorandil-containing chow from day 2 (STZ-DM-N48), 3 (STZ-DM-N72), and 4 (STZ-DM-N96) to day 30. Body weight, blood glucose, and plasma insulin were measured every week. For the in vitro assay, neonatal rat islet-rich cultures were performed and cells were treated with nicorandil from 1 h before to 2 h after exposure to STZ for 30 min. Insulin secretion from islet cells was assayed after an additional 24 h of culture. We also observed the effect of nicorandil on the generation of reactive oxygen species (ROS) from rat insulinoma cells (RINm5F).

Results: Body weight loss and blood glucose levels of STZ-DM-N48 rats were significantly lower than those of STZ-DM rats. Immunohistochemical staining of insulin showed preservation of insulin-secreting islet β-cells in STZ-DM-N48 rats. Nicorandil also dose-dependently recovered the insulin release from neonatal rat islet cells treated with STZ in in vitro experiments. Nicorandil did not act as a PCO on neonatal rat islet β-cells or RINm5F cells, and did not show an inhibitory effect on poly(ADP-ribose) polymerase-1. However, the drug inhibited the production of ROS stimulated by high glucose (22.0 mmol/l) in RINm5F cells.

Conclusions: These results suggested that nicorandil improves diabetes and rat islet β-cell damage induced by STZ in vivo and in vitro. It protects islet β-cells, at least partly, via a radical scavenging effect.

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Introduction

Type 1 diabetes results from the destruction of insulin-producing β-cells by a cytotoxic T cell-mediated antigen-specific process (1, 2). An increased expression of cytokines in association with insulitis has been reported, and different combinations of cytokines have been shown to decrease the function and viability of β-cells in vitro, partially through the production of nitric oxide and reactive oxygen species (ROS) by the β-cells themselves or by immune cells near or in the islets (3). The development of type 2 diabetes is usually associated with a combination of β-cell dysfunction and insulin resistance. Once hyperglycemia becomes apparent, β-cell function deteriorates (4). Under diabetic conditions, ROS are produced mainly through glycation reaction (5, 6), which occurs in various tissues (7) including pancreatic islets and may play a role in the development of complications and further destruction of β-cells (8–10).

It has been reported that the anti-oxidants, N-acetyl-L-cysteine, vitamin C, and vitamin E, or a combination of these drugs, protect the islet β-cells in diabetic animal models (11). It has also been demonstrated in vivo that overexpression of the anti-apoptotic, anti-oxidant protein thioredoxin (12), catalase (13), or metallothionein (14) protects the β-cells from streptozotocin (STZ). However, gene transduction in islet β-cells after the onset of diabetes in vivo is difficult.

N-[2-hydroxyethyl]-nicotinamide nitrate (nicorandil) is a hybrid drug comprising moieties of a cardiac ATP-sensitive K⁺ channel opener (PCO) and a nitrate (15). The drug is clinically available and currently being used especially in Europe and Japan for the treatment of angina (16, 17). In addition, nicorandil may also possess anti-free-radical characteristics, since the
nicotinamide moiety of its molecular structure is a known hydroxyl radical scavenger. This drug has been reported to show a hydroxyl-radical scavenging effect and an inhibitory effect on superoxide anion production (18, 19). In this study, we have investigated the protective action of nicorandil on STZ-induced β-cell damage in vivo and in vitro.

Materials and methods

Animals

Six-week-old male Brown Norway rats weighing approximately 190 g and newborn (3- to 4-day-old) Wistar rats were used for these experiments. All animals were maintained in a specific pathogen-free environment on a 12 h light:12 h darkness cycle. All procedures adopted and experiments undertaken followed the principles of laboratory animal care as described by the NIH.

In vivo studies

Brown Norway rats (n = 25) received a single 65 mg/kg intraperitoneal injection of STZ (Sigma Chemical Co., St Louis, MO, USA) in 10 mmol/l sodium citrate buffer, pH 4.5. Control, non-diabetic rats (n = 8) received citrate buffer alone. Diagnosis of diabetes was confirmed by blood glucose level above 14 mmol/l (Glucocard GT-1640 and Diasensert strips; Arkray, Kyoto, Japan) 48 h after STZ administration. At that time, the diabetic rats (n = 23) were divided into four groups. Rats in the control and STZ-induced diabetic (STZ-DM) (n = 6) groups were kept on a regular diet without nicorandil. The other three groups were given 0.003% nicorandil-containing diet from 2 (STZ-DM-N48) (n = 6), 3 (STZ-DM-N72) (n = 6), and 4 (STZ-DM-N96) (n = 5) days after the injection of STZ. Two rats in the STZ-DM group, one rat in the STZ-DM-N48 group, and one rat in the STZ-DM-N-72 group died during the in vivo experiment. Blood samples were obtained from the tail veins of rats in the non-fasting state. Blood glucose and insulin levels (Mercodia rat insulin ELISA; Mercodia AB, Uppsala, Sweden) were measured every 7 days in each group. All rats were killed 30 days after STZ administration, and each pancreas was stored in Bouin’s solution for immunohistochemistry.

Neonatal rat pancreatic cell and RINm5F cell culture

Rat pancreases were isolated from 3- to 4-day-old Wistar rats. Eight to ten pancreases were minced into pieces smaller than 1 mm³. After washing in RPMI1640 (Gibco, Carlsbad, CA, USA), the fragments were digested for 15 min in a small flask using starter in 10 ml Hank’s balanced saline solution containing 0.2% trypsin (DIFCO Laboratories, Detroit, MI, USA) and 2 mg/ml collagenase type 1 (Worthington, Lakewood, NJ, USA) at 37 °C. After removal of the supernatant, the digestion was repeated for 15 min. To obtain isolated cells or small clusters of cells, gentle pipetting using a Pasteur pipette was repeated. The pancreatic cells were centrifuged in RPMI1640 at 300 g for 10 min. The cells were resuspended and plated on 24-well culture dishes at a concentration of one pancreas per plate in RPMI1640 containing 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 16.7 mmol/l glucose at 37 °C in humidified air with 5% CO₂. Rat insulinoma cells, RINm5F, purchased from the American Type Culture Collection (CRL-2058; Manassas, VA, USA), were plated on 24-well dishes at a concentration of 2 × 10⁷ cells/well. The cells were incubated in RPMI1640 containing 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 11.0 mmol/l glucose at 37 °C in humidified air with 5% CO₂. All the experiments using RINm5F were performed after 48–72 h culture.

Insulin secretion

After 3 days of neonatal rat pancreatic cell culture, the new culture media containing 16.7 mmol/l glucose, stock solutions of 100 mmol/l nicorandil (purchased from Chugai Pharmaceuticals, Tokyo, Japan), or diazoxide (Sigma) dissolved in dimethyl sulfoxide (DMSO) were added to reach a final concentration of 30–300 μmol/l in the culture dishes. In some experiments, nitrol was used as a nitric oxide donor in the same range of concentrations. In all media, final concentrations of DMSO were adjusted to 0.3%. Cells were incubated in 5% CO₂ at 37 °C for 1 h with or without these drugs, then STZ was added to the wells to give a final concentration of 2 mmol/l. The incubation continued for an additional 30 min, and was terminated by washing with cold RPMI1640 medium. The cells were then incubated for 2 h in new culture media containing the same concentrations of nicorandil, diazoxide, or nitrol. After washing the cells, the culture was continued for an additional 24 h in the drug-free culture media. The cells were then washed twice and incubated in 1 ml RPMI1640 containing 0.2% bovine serum albumin (BSA) and 16.7 mmol/l glucose for 1 h. Insulin concentrations in the media were measured, standardized by the amount of protein, and are described as % of control. To observe the effects of drugs on basal insulin secretion, rat pancreatic cells or RINm5F cells were washed with phosphate-buffered saline (PBS) and cultured with RPMI1640 containing 0.2% BSA and the concentrations of glucose indicated for 3.5 h in the presence of various concentrations of nicorandil, diazoxide, or nitrol.

Immunohistochemical and immunofluorescent staining

For the staining of rat pancreases, primary antibodies specific for insulin (diluted 1:200; polyclonal anti-insulin
guinea pig IgG; Oriental Yeast Co, Ltd, Tokyo, Japan) and glucagon (diluted 1:200; clone K79bB10; Sigma) were applied to 4 μm paraffin-embedded serial sections obtained from at least two pancreases in each group. As second antibodies, HRP-conjugated goat anti-guinea pig IgG or HRP-conjugated anti-mouse IgG were used. Substrate chromogen (Envision System: Dako, Glostrup, Denmark) was added for 45 s and washed with PBS. Stained islets were observed by photomicroscope.

For the immunofluorescent staining of insulin in rat pancreatic cell cultures, cells were plated on 6 cm dishes at the same density as that of the 24-well cultures. After 3 days of culture, cells were washed and fixed with Bouin’s solution for 10 min. Cells were permeabilized by PBS containing 0.1% triton X-100 for 10 min, and washed with PBS. Next, primary antibodies specific for insulin (diluted 1:200) were added to the cells for 30 min, after which they were washed with PBS. Fluorescein isothiocyanate-labeled rabbit anti-guinea pig IgG (diluted 1:200) was added for 10 min, and washed with PBS. Stained insulin-containing cells were observed by fluorescence microscopy.

**ROS assay**

The intracellular formation of ROS species was measured using 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) (Sigma). The non-fluorescent compound DCFH-DA penetrated into the cells, and was cleaved by intracellular esterases, resulting in the formation of 2′,7′-dichlorodihydrofluorescein (DCFH). The oxidation of DCFH on oxidative stress generates the highly fluorescent compound dichlorofluorescein.

After changing the culture media of RINm5F cells, various concentrations of nicorandil, diazoxide, or nitrofur were added. Cells were incubated in 5% CO₂ at 37 °C for 1 h with or without these drugs, then the media were changed and the concentrations of glucose (5.5 or 22.0 mmol/l) adjusted. The incubation continued for an additional 8 h in the presence of drugs, and was terminated by washing with cold RPMI1640 medium. The cells were then loaded with 20 μmol/l DCFH-DA for 30 min. After washing with PBS, cells were analyzed by Fluoroskan Ascent CF (MTX Labsystems, Inc., Vienna, VA, USA) with excitation and emission settings of 495 and 538 nm respectively. Fluorescence intensity was standardized by the amount of total protein in each well.

**Poly(ADP-ribose) polymerase-1 (PARP-1) inhibition assay**

The effects of nicorandil and nicotinamide on PARP-1 were assayed using a PARP inhibition assay kit (No. 4669-96-K; Trevigen, Inc., Gaithersburg, MD, USA) according to the manufacturer’s instructions. Stock solutions of 100 mmol/l nicorandil and nicotinamide dissolved in DMSO were diluted with assay buffer. DMSO alone was also diluted with assay buffer as control samples (vehicle). 3-Aminobenzamide was used as a positive inhibitor of PARP-1. Inhibition of PARP-1 activity reduces optical density (OD; 630 nm) values.

**Statistical analysis**

Statistical analysis was performed using Student’s t-test. All data are presented as means±S.E.M., and statistical significance was achieved when P < 0.05.

**Results**

**In vivo studies**

Figure 1 shows blood glucose levels, plasma insulin levels, and body weight of the five groups of rats. Before the experiments (day 0), there was no significant difference in any of the parameters among these groups (data not shown). Glucose and insulin levels in diabetic rats were not different among groups at the onset of diabetes (day 2). Blood glucose levels of STZ-DM rats gradually increased, and reached 27.3±2.9 mmol/l on day 30. Each nicorandil-treated group showed some reduction in the glucose level compared with that in STZ-DM rats (Fig. 1A). In nicorandil-treated groups, glucose levels of STZ-DM-N48 rats did not increase after the onset of diabetes. Blood glucose levels on day 2 and day 30 were 18.7±2.1 and 17.8±2.9 mmol/l respectively. Blood glucose levels of the other nicorandil-treated groups, STZ-DM-N72 and STZ-DM-N96, were increased to 22.2±1.2 and 24.5±1.6 mmol/l respectively on day 30. On day 16 and day 30, glucose levels of STZ-DM-N48 rats were significantly lower than those of STZ-DM or STZ-DM-N96 rats. These results indicated that nicorandil treatment after the onset of STZ-induced diabetes attenuated the progression of diabetes, and the effects of nicorandil were more potent when administration of the drug was started at an early stage of diabetes.

We also measured body weight (Fig. 1B) and plasma insulin levels (Fig. 1C). Plasma insulin concentrations were higher in STZ-DM-N48 than in STZ-DM rats. However, the difference was not significant. Body weight in the control group was gradually increased from 218±6.6 g on day 2 to 277±4.5 g on day 30. Body weight of STZ-DM rats was 212±14.6 g on day 30. Rates of increase in the body weight of nicorandil-treated groups were low compared with that of non-diabetic controls. However, nicorandil treatment, especially in the STZ-DM-N48 group, significantly increased body weight. On day 30, body weights of the control, STZ-DM, STZ-DM-N48, STZ-DM-N72, and STZ-DM-N96 rats were 277±4.5, 212±14.6, 259±6.8, 226±12.8, and
216±9.9 g respectively. The body weight of STZ-DM-N48 rats was significantly higher than that of STZ-DM or STZ-DM-N96 rats.

**Immunohistochemical staining for insulin and glucagon**

Figure 2 shows immunostaining for glucagon (Fig. 2A, C and E) and insulin (Fig. 2B, D and F). In the control pancreas, islets were clearly stained with both anti-insulin and anti-glucagon antibodies. The photographs show the normal structure of the islet composed of insulin-containing cells surrounded by marginal glucagon-containing cells (Fig. 2A and B). Consistent with plasma insulin concentrations in these rats on day 30, insulin content in the islets of STZ-DM-N48 rats was found to be partly retained compared with that of STZ-DM rats (Fig. 2F). However, the staining pattern of the islets in STZ-DM-N48 rats was irregular, and there were many small cell clusters stained only with anti-glucagon antibody. In STZ-DM rats, the number of insulin-containing cells was remarkably decreased. The staining intensity of glucagon was also decreased in this group. We randomly selected ten islets in the control, STZ-DM, and STZ-DM-N48 groups, and obtained the area stained with anti-insulin and anti-glucagon antibodies. The calculated ratio of the area composed of β-cells to that composed of α-cells in these groups were 1.31±0.10, 0.06±0.01, and 0.12±0.03 respectively. The ratio of β-cell area in STZ-DM-N48 rats was significantly (P < 0.05) higher than that in STZ-DM rats.

**Effects of drugs on insulin release from neonatal rat islet cells**

After 3 days of culture, rat neonatal pancreatic cell culture showed many insulin-containing cells or islet-like cell clusters (Fig. 3). The cells were then washed with PBS and cultured with RPMI1640 containing 0.2%
BSA and 16.7 mmol/l glucose in the presence of various concentrations of nicorandil, diazoxide, or nitrol for 3.5 h. Diazoxide dose-dependently inhibited the secretion of insulin from rat islet cells. The drug decreased the insulin release to 74.2±7.6% (30 μmol/l), 46.4±3.3% (100 μmol/l), and 51.3±8.2% (300 μmol/l) of the control level. Nicorandil and nitrol had no inhibitory effect on the secretion of insulin (Fig. 4). These results suggested that nicorandil (30–300 μmol/l) had no PCO activity on rat islet β-cells.

Protection of STZ-induced damage of pancreatic islets by nicorandil in vitro

Thirty minutes of exposure of rat pancreatic cells to STZ (2 mmol/l) abolished ~70% of insulin release from rat pancreatic islets. Nicorandil and diazoxide showed protective effects on the function of insulin secretion (Fig. 5). Nicorandil dose-dependently increased insulin secretion. The release of insulin recovered from 29.2±2.90% (drug free) to 39.0±4.1% (30 μmol/l), 43.8±3.3% (100 μmol/l), and 50.0±5.33% (300 μmol/l). The differences between the nicorandil-free group and each of the nicorandil-treated groups were significant. Diazoxide also dose-dependently increased insulin secretion from 34.5±2.84 (drug free) to 33.4±3.9% (30 μmol/l), 39.8±3.7% (100 μmol/l), and 54.6±3.19% (300 μmol/l). Nitrol showed no protective effect on insulin release from rat islets damaged by STZ.
**ROS assay**

We next examined the effect of nicorandil on ROS generation in the rat islet-like cells, RINm5F. Thirty minutes of exposure of neonatal rat pancreatic cells or RINm5F cells to 2 mmol/l STZ did not induce statistically significant up-regulation of ROS production detected by DCFH-DA (data not shown). We therefore added high glucose (22.0 mmol/l) to increase ROS production in RINm5F cells. High glucose treatment for 8 h increased ROS production in the cells. Nicorandil slightly decreased ROS generation stimulated by high glucose (Fig. 6A). In the same experiments, nicorandil did not inhibit insulin secretion from RINm5F cells, and diazoxide significantly inhibited insulin secretion (Fig. 6B). However, diazoxide had no inhibitory effect on ROS production (Fig. 6A).

**PARP-1 inhibition assay**

PARP-1 is the enzyme catalyzing the nicotinamide adenine dinucleotide (NAD)-dependent addition of ribose to adjacent nuclear proteins. PARP-1 plays an important role in DNA repair but can also lead to cell death by depleting the cellular NAD pool. Therefore, nicotinamide, a PARP-1 inhibitor, protects islet β-cells damaged by STZ (20). To investigate whether the mechanism of action by nicorandil may depend on an inhibition of PARP-1 activity, we observed the effects of nicotinamide and nicorandil on the enzyme (Fig. 7). In this experiment, control positive PARP-1 inhibitor (3-aminobenzamide) and nicotinamide dose-dependently inhibited PARP-1. More than 100 μmol/l (0.1 mmol/l) of both drugs remarkably reduced the OD (630 nm) values. However, nicorandil did not show any inhibitory effect.
DMSO were diluted with assay buffer. Vehicle (solutions of 100 mmol/l nicorandil and nicotinamide dissolved in control samples) was diluted with assay buffer, and these buffers were used as control samples.

**Discussion**

In the present study, we found that nicorandil improved STZ-induced diabetes mellitus in vivo and protected β-cells against the toxic action of STZ in vitro. Our in vivo data showed that 4 weeks of oral treatment with nicorandil to STZ-DM rats resulted in decreased blood glucose levels, retained plasma insulin concentrations, preservation of islet β-cells, and prevention of body weight loss compared with rats in the STZ-DM group that did not receive nicorandil treatment.

STZ is an agent widely applied to cause experimental diabetes because of its ability to selectively target and destroy insulin-producing pancreatic islet β-cells (21). The glucose moity of STZ allows preferential uptake of the toxin into β-cells via the glucose transporter, GLUT-2 (22, 23). STZ is an alkylation agent and causes DNA strand breaks, which then activate poly(ADP-ribose) synthetase. Activation of poly(ADP-ribose) synthetase induces rapid and lethal depletion of NAD in β-cells (20, 24). Its diabetogenic action has also been ascribed to production of nitric oxide (25, 26) and ROS (27, 28). We originally investigated the inhibitory effects of nicorandil and gliclazide on leukocyte activation (29) and the adhesion of leukocytes in the retinal microvessels in severe diabetic rats induced by STZ (30). In these experiments, we unexpectedly found that administration of nicorandil decreased the blood glucose level in STZ-induced diabetic rats.

Consequently, we undertook to investigate the therapeutic effect of nicorandil in diabetic rats. It has recently been reported that diazoxide and its analog (NNC 55-0118) protect STZ-treated rat islets via PCO activity (31, 32). These drugs activate the pancreatic ATP-sensitive K⁺ channel and hyperpolarize the β-cells, resulting in inhibition of the energy-consuming process of insulin secretion. However, the PCO action of nicorandil on islet β-cells cannot explain the therapeutic effect of the drug in STZ-induced diabetes. The affinity of nicorandil for the pancreatic ATP-sensitive K⁺ channel, SUR2.kir6.2, is very weak (33), and inhibition of insulin secretion might induce further hyperglycemia leading to more severe stress on β-cells in vivo, because we had treated STZ-induced diabetic rats with nicorandil alone (i.e., without insulin injection). In our in vivo experiments, nicorandil treatment was started after the onset of STZ-induced diabetes. Hence, STZ had already destroyed almost all the islet β-cells before nicorandil treatment. The small number of residual β-cells were exposed to high glucose after the onset of diabetes mellitus. It is speculated that STZ acutely destroyed the majority of islet β-cells, and, in the next step, high glucose gradually impaired the residual β-cells. In both steps, free radicals may play a part in the degeneration of β-cells in vivo.

To clarify the action mechanisms of nicorandil for β-cell protection, we then performed an in vitro study using pancreatic cell culture or a rat insulinoma cell line. We observed the effect of nicorandil on STZ-treated islet cells. Nicorandil appeared to be as potent as diazoxide, a strong cardiac and pancreatic PCO, in protecting islet β-cells against STZ-induced damage in vitro. According to a previous report, diazoxide caused a strong dose-dependent inhibition of insulin release from mouse islets (93% at 100 μmol/l) (34). However, nicorandil was reported to be less effective (25% at 500 μmol/l) (35). We also confirmed that nicorandil (30–300 μmol/l) did not inhibit the secretion of insulin from rat islet β-cells. Nicorandil also acts as a nitrate as well as a cardiac PCO. The strong nitric oxide donor, nitrol (30–300 μmol/l), did not show an inhibitory effect on insulin release or a protective effect on islet β-cells damaged by STZ. Therefore, PCO or a nitrate-like action does not explain the protective effect of nicorandil on β-cells.

As described earlier, STZ enters the cytoplasm via GLUT2. The presence of this transporter may account in part for the specific vulnerability of β-cells to STZ treatment. However, GLUT2 is also present in the liver and kidney, both of which are relatively resistant to STZ damage. The much greater sensitivity of the β-cell is probably due to its very low level of anti-oxidant enzyme expression and activity (36–38), which leaves it unable to inactivate ROS. We found that high glucose (22.0 mmol/l) for 8 h of cultivation increased ROS production in RINm5F cells. Nicorandil slightly inhibited high glucose-induced ROS production. In these examinations, diazoxide did not decrease ROS.
production at any of the concentrations tested. One of the other possible action mechanisms of nicorandil on islet β-cell protection is a PARP-1 inhibitory effect. Nicorandil has both nitrate and nicotinamide moieties, and nicotinamide has been reported as a PARP-1 inhibitor. PARP-1 knockout mice and mice treated with nicotinamide are protected against the STZ-induced diabetogenic effect (20, 39). In our experiment, nicorandil did not show an inhibitory effect on PARP-1. This result indicated that the protective mechanisms of nicorandil and nicotinamide on islet β-cells damaged by STZ might be different.

The findings of our in vivo and in vitro studies have suggested that nicorandil improved STZ-induced diabetes via a radical scavenging effect rather than via inhibition of energy consumption of β-cells achieved by PCO action or PARP-1 inhibition.

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