Antihypertensive effect of insulin via nitric oxide production in the Zucker diabetic fatty rat, an animal model for non-insulin-dependent diabetes mellitus

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Abstract

It has been reported that insulin treatment improves hypertension in patients with diabetes mellitus. The mechanisms of the antihypertensive effect of insulin, however, remain to be fully elucidated. In the present study, we investigated a possible involvement of nitric oxide (NO) in insulin-induced reduction of blood pressure using the Zucker diabetic fatty (ZDF) rat, an animal model of non-insulin-dependent diabetes mellitus. The animals were divided into three groups and treated for 4 weeks with daily subcutaneous injections of insulin (25 U/kg body weight) with or without oral administration of L-nitro-arginine methyl ester (L-NAME, 50 mg/kg/day body weight as drinking water), an inhibitor of NO synthase (NOS). Saline solution was injected subcutaneously in the control groups. During the experimental period, body weight gain was greater in the insulin-treated groups than in the control groups whereas water intake was considerably decreased in the insulin-treated groups. Insulin treatment resulted in a decrease in plasma glucose and blood pressure, and an increase in both NO metabolites (NOx) in the plasma and NOS activity in the aorta tissue. L-NAME treatment blunted not only the antihypertensive effect of insulin but also the changes in NOx and NOS activity. These findings suggest that insulin reduces blood pressure in the ZDF rat by stimulating NOS activation and NO production.

European Journal of Endocrinology 140 341–349

Introduction

Hypertension is one of the complications in diabetes mellitus (DM) and is considered to be a risk factor for such serious vascular episodes as myocardial infarction and cerebral infarction in diabetic patients (1, 2). Insulin has vasodilative and hypotensive effects (3–9). It has been reported that hypertension was improved by insulin treatment in patients with DM (10–12). The antihypertensive effect of insulin was explained either by the secondary action of improved glucose metabolism after insulin treatment or by the direct action on peripheral arterial vessels (4, 10–16). Peripheral arterial resistance is a critical factor for the regulation of diastolic blood pressure, which is increased by atherosclerosis or vasoconstriction (17–20).

Recently, nitric oxide (NO) has been identified as an endothelial derived relaxing factor which acts as a modulator of vascular resistance under physiological conditions (21, 22). Several lines of evidence suggest that insulin stimulates NO generation in endothelial cells (23–25). Clinically, NO metabolites in plasma and urine increased in diabetic patients treated with insulin (26–29). These results raise the possibility that the

antihypertensive effect of insulin is closely related to endothelial NO generation. To clarify this possibility, we examined the antihypertensive effect of insulin in Zucker diabetic fatty (ZDF) rats, in which obesity, hyperglycemia and hyperinsulinemia are genetically expressed at a young age (30). Hypertension occurs after the diabetic state develops in the ZDF rat. Thus, hypertension is considered to be a major vascular complication of DM in the ZDF rat, a model of non-insulin-dependent diabetes mellitus (NIDDM).

In the present study, to investigate the mechanism of antihypertensive effects of insulin, we studied the effects of insulin on plasma glucose, blood pressure and NO generation in ZDF rats. We report our findings suggesting that insulin improves hypertension in DM by stimulating NO synthase activation and NO production.

Materials and methods

Drugs

Recombinant human insulin (Novolin N) was purchased from Novo Nordisk (Bagsvaerd, Denmark).

Guinea pig antiserum to porcine insulin was generated in our laboratory. L-Nitro-arginine methyl ester (L-NAME) was purchased from Aldrich Chemical (Mikwaukee, WI, USA). L-[^3H]Arginine (2.33 GBq/mmol) and L-[^{14}C]citrulline (2.2 GBq/mmol) were purchased from Amersham Life Science (Amersham, Bucks, UK) and New England Nuclear (Boston, MA, USA) respectively. Tetrahydrobiopterin was a generous gift from Suntory Biomedical Research Center, Osaka, Japan. All other chemicals were of the purest grade available from regular commercial sources.

Animals

Male ZDF rats were obtained at the age of 7 weeks from Genetic Models Inc. (Indianapolis, IN, USA), housed in pairs, and kept in an artificial lighting regime (lights on at 0800 h, off at 2000 h). Food and tap water were freely given until the age of 12 weeks when the animals were divided into three groups. In the first and second groups, insulin therapy was started at the age of 12 weeks when plasma glucose levels were over 200 mg/ml. Insulin was injected subcutaneously in a dose of 25 U/kg/day between 1700 h and 1800 h at 24-h intervals during the next 4 weeks as previously described (31). The second group was also treated with L-NAME, which was administered to drinking water in a dose of 50 mg/kg/day after the age of 12 weeks. In the third group (control group), saline solution was injected subcutaneously in a dose of 1.25 ml/kg/day.

After 9 weeks of age, 0.5 ml blood was collected from the tail vein without anesthesia between 0900 and 1100 h every week as previously described (32). Plasma was obtained by centrifugation at 3000 r.p.m. for 10 min at 4 °C. Plasma glucose and insulin levels were measured by the glucose oxidase method using Fuji Dry Kem (Fuji Film, Minami Ashigara, Japan), and a specific radioimmunoassay with a rat insulin standard (a generous gift from Eli Lilly and Company, Indianapolis, IN, USA) and guinea pig antiserum to porcine insulin (33). Plasma levels of NO metabolites and osmolarity were measured as previously described (34, 35). During the experimental period, food and water intake as well as body weight were monitored at 1800 h each day. Systolic blood pressure was monitored by the tail-cuff method. After 4 weeks of treatment, all the animals were decapitated and the whole aorta, defined by the aortic arch through the bifurcation of the iliac artery, was removed, immediately frozen in liquid nitrogen and stored at -80 °C until the NO synthase (NOS) activity was assayed.

Animal experiments were performed in accordance with the procedures outlined in the Guidelines for Animal Experimentation of Shimane Medical University, which complied with the Guidelines for Animal Experimentation of the Japanese Association for Laboratory Animal Science.

Nitrite (NO_2^-) and nitrate (NO_3^-) assay

For the measurement of NO metabolites in rat plasma, 250 μl plasma were diluted with the same volume of the mobile phase of the NOx ($\text{NO}_2^- + \text{NO}_3^-$) analysis system (10% methanol containing 0.15 mol/l NaCl- NH_4Cl and 0.5 g/l EDTA4Na) and deproteinized by ultrafiltration with microcon 30 (Amicon, Beverly, MA, USA) at 15 000 r.p.m. at 4 °C for 60 min and the resulting filtrate was stored at -20 °C until assayed.

Nitrite and nitrate concentrations were measured by the NOx analyzing HPLC system (ENO-10, EICOM, Kyoto, Japan). The analysis system consists of a pump for the mobile phase (10% methanol containing 0.15 mol/l NaCl- NH_4Cl and 0.5 g/l EDTA4Na, at a

Figure 1 Effects of insulin and L-NAME on food, water intake and body weight in ZDF rats. Food (A), water intake (B) and body weight (C) were monitored at 1800 h every day. Each value shows the mean $\pm$ S.E.M. of 5 animals examined. (○), Control group; (▲), insulin-treated group; (■), insulin and L-NAME-treated group. * $P < 0.05$, ** $P < 0.01$ vs the control group.

flow rate of 0.33 ml/min), a pump for the Griess reagents (1.25% HCl containing 5 g/l sulfanilamide and 0.25 g/l N-(1-naphthyl)-ethylenediamine, at a flow rate of 0.1 ml/min), a reverse phase separating column (NO-PAK, 4.6 mm×50 mm, EICOM), a Cd reducing column (NO-RED, EICOM) and a flow-through spectrophotometer (NOD-10, EICOM). Samples were kept cool and applied to the analysis system by an automated sample injector (AS-8020, Tosoh, Tokyo, Japan). After nitrite and nitrate were separated by a separating column, nitrate was reduced to nitrite by a Cd reducing column. Then, nitrite was mixed with Griess reagents and a reaction product was measured by a flow-through spectrophotometer at 540 nm. Prior to each experiment, all the tubes and tips for the pipette were washed with purified water prepared by Milli-Q Labo (Millipore, Bedford, MA, USA) to reduce contamination of nitrite and nitrate. NaNO₂ and NaNO₃ were used as authentic standards.

NOS activity assay

Rat aortic tissue was homogenized with 10 volumes 50 mmol/l Tris-HCl (pH 7.4) containing 1 mmol/l dithiothreitol, 1 mmol/l EDTA, 10 µg/ml leupeptin, 10 µg/ml aprotinin and 1 mmol/l phenylmethylsulfonyl fluoride and sonicated twice for 10 s each. Then the homogenate was centrifuged at 100 000 *g* for 30 min at 4 °C. The resulting supernatant was stored at -80 °C until assayed.

NOS activity was measured by production of L-[³H]citrulline from L-[³H]arginine. An aliquot (50 µl) of the supernatant was incubated at 37 °C for 20 min in the presence of 50 mmol/l Tris-HCl (pH 7.5), 1 mmol/l NADPH, 10 µmol/l flavin adenine dinucleotide, 10 µmol/l tetrahydrobiopterin, 40 µmol/l L-arginine, 3.7 MBq L-[³H]arginine and 4 mmol/l CaCl₂ in a total volume of 100 µl. The reaction was halted by the addition of 1.25 ml ice-cold 20 mmol/l HEPES (pH 5.5), and the total volume was applied on a Dowex-50 W×8

Figure 2 Effects of insulin and L-NAME on plasma insulin and plasma glucose levels in ZDF rats. Blood samples were obtained from the tail vein without anesthesia in the morning each week and plasma was immediately collected for analysis of insulin and glucose levels. Each value shows the mean $\pm$ S.E.M. of 5 animals examined. (○), Control group; (▲), insulin-treated group; (■), insulin and L-NAME-treated group. (A) Effects of insulin and L-NAME on plasma insulin levels in ZDF rats. * P < 0.01 vs the control group. (B) Effects of insulin and L-NAME on plasma glucose levels in ZDF rats. * P < 0.05, ** P < 0.01 vs the control group.

(200–400 mesh, Na^+ form) column pre-equilibrated with 20 mmol/l HEPES (pH 5.5). $\text{L-}[^3\text{H}]\text{Arginine}$ was retained on the column, whereas $\text{L-}[^3\text{H}]\text{citrulline}$ was recovered in the effluent. Therefore, we collected the follow-through and 2 ml of the distilled water wash, and the radioactivity was measured by a liquid scintillation counter. The recovery of citrulline was determined by applying $\text{L-}[^{14}\text{C}]\text{citrulline}$ solution (80–90%). The concentration of $\text{L-}[^3\text{H}]\text{citrulline}$ produced was corrected for recovery.

NADPH diaphorase staining

After treatment for 4 weeks, the rats were anesthetized with intraperitoneal administration of sodium pentobarbital (50 mg/kg) and perfused with 300 ml 0.9% saline followed by 600 ml 4% paraformaldehyde in 0.1 mol/l phosphate buffer (pH 7.4) via the left ventricle. The whole aorta, defined by the aortic arch through the bifurcation of the iliac artery, was removed and immersed in the same fixative at 4 °C overnight. After washing with 0.1 mol/l phosphate buffer three times, one half of the aorta was cryoprotected at 4 °C overnight by gradually increasing the sucrose concentrations by 10%, 20% and 30% at 24-h intervals. Then the aortic tissue was embedded in OCT compound and

frozen in liquid nitrogen. Frozen sections (20 μm) were cut using a cryostat (coldtome, model CM-41, Sakura, Tokyo, Japan) and placed on poly-L-lysine-coated glass slides. Aortic sections were incubated at 4 °C in the dark overnight in 0.1 mol/l phosphate buffer containing 1.2 mmol/l $\beta\text{-NADPH}$ as a reduced form, 0.3 mmol/l nitro blue tetrazolium and 0.2% Triton X-100. The reaction was terminated by washing the sections with ice-cold 0.1 mol/l phosphate buffer (pH 7.4). Sections were then cover-slipped with glycerol. Several sections were then dehydrated by ethanol and embedded in paraffin. Slices (5 μm) were prepared for hematoxylin-eosin (HE) staining by the use of a rotary microtome (RM 2135, Leica, Heidelberg, Germany) as previously described (36, 37).

Statistical analysis

All results are expressed as means $\pm$ S.E.M. The statistical differences were evaluated with ANOVA and Fisher's test. A probability level of $P < 0.05$ was considered significant.

Results

Food intake was constantly preserved during the experimental period in the control group. In the

Figure 3 Effects of insulin and L-NAME on systolic blood pressure and plasma NOx concentrations in ZDF rats. Each value shows the mean $\pm$ S.E.M. of 5 animals examined. (○), Control group; (▲), insulin-treated group; (■), insulin and L-NAME-treated group. (A) Effect of insulin on systolic blood pressure in ZDF rats. Systolic blood pressure was measured by the tail cuff method in the morning each week. (B) Effect of insulin on plasma NOx levels in ZDF rats. Blood samples were obtained from the tail vein without anesthesia in the morning each week and plasma was ultrafiltrated for analysis of NO_2^- and NO_3^- . Plasma NOx concentrations were measured by the NOx analysis system as described in the text. * $P < 0.05$, ** $P < 0.01$ vs the control group. # $P < 0.05$, ## $P < 0.01$ vs the insulin-treated group.

insulin-treated group and the group treated with insulin and L-NAME, food intake was similar until 3 weeks after the start of the treatment, but it was lower in the latter group at 4 weeks after the start of treatment. Water intake markedly decreased and remained low until the end of the experiment in both the insulin-treated group and the insulin and L-NAME-treated group as compared with the control group (Fig. 1A, B). The body weight of the insulin-treated rats was much greater one week after the administration of insulin and remained greater until the end of the experiment. The body weight of the insulin and L-NAME-treated rats was also greater two weeks after the start of treatment and remained greater in a similar manner to the insulin-treated rats. There was no statistical difference in the body weight between the insulin-treated rats and the insulin and L-NAME-treated rats (Fig. 1C).

Plasma insulin levels markedly increased after the injection of insulin both in insulin-treated rats and in insulin and L-NAME-treated rats. The plasma insulin level in the control group remained fairly constant from the 12th week until the end of the experiment (Fig. 2A). Plasma glucose levels decreased after the administration of insulin both in the insulin-treated rats and in the insulin and L-NAME-treated rats. The effect of insulin on plasma glucose was independent of L-NAME. In both groups, the plasma glucose level remained low until two weeks after the administration of insulin, then the plasma glucose level gradually increased even in the presence of insulin. However, the plasma glucose level was significantly lower in both the insulin-treated rats and the insulin and L-NAME-treated rats compared with the control rats during treatment with insulin (Fig. 2B).

Compared with the control group, systolic blood pressure decreased in the insulin-treated rats two weeks after the start of insulin administration and remained low until the end of the experiment. Systolic blood pressure was greater in the insulin and L-NAME-treated rats than in the control group one week after the start of insulin administration, and remained higher until the end of the experiment except at two weeks after insulin administration (Fig. 3A).

Plasma NO_x levels increased during the treatment with insulin whereas simultaneous administration of L-NAME blunted the insulin-induced increase in plasma NO_x and reduced plasma NO_x levels compared with the control group (Fig. 3B).

NOS activity in the aortic tissue remarkably increased in the group treated with insulin whereas it was not considerably changed in the insulin and L-NAME-treated group and in the control group (Fig. 4).

HE staining revealed that the smooth muscle layer of the aorta in the insulin-treated group was apparently thin due to aortic dilatation and that the aortic smooth muscle layer of the insulin and L-NAME-treated group was apparently thick due to aortic constriction compared with the control group (Fig. 5A, B and C). NADPH diaphorase staining showed that the endothelium and

the smooth muscle in the aorta were more densely stained in the insulin-treated group than in the control group. Among the three groups these areas were most densely stained in the insulin and L-NAME-treated group (Fig. 5D, E and F).

Discussion

In the present study we have clearly demonstrated that insulin treatment improved hypertension associated with DM possibly via stimulated NOS activation and NO production.

In the ZDF rats, increased plasma glucose levels were evident at the age of 12 weeks, when insulin treatment was started. Although the dose of insulin administered increased according to the body weight gain in these animals, plasma glucose levels gradually increased. This may be due to an increase in the resistance to insulin since plasma insulin levels were threefold higher in the ZDF rat than those in the Lean rat, a non-diabetic phenotype for the ZDF rat, at the age of 9 weeks, and remained high at the age of 16 weeks (data not shown). It is likely that the insulin-induced increase in body weight was caused by improved metabolism which induces a decrease in water intake and an accumulation of fat in the subcutaneous tissue.

Increases in body weight, plasma glucose levels and insulin resistance in ZDF rats are compatible with clinical findings in obese patients with NIDDM. In the

Figure 4 Effects of insulin and L-NAME on NOS activity in the aorta of ZDF rats. At the end of the experimental period, the aorta was removed and homogenized for analysis of NOS activity. NOS activity was measured by production of L-[³H]citrulline from L-[³H]arginine as described in the text. Each value shows the mean $\pm$ S.E.M. of 4 animals examined. ** $P < 0.01$ vs the control group. ## $P < 0.01$ vs the insulin-treated group.

Figure 5 Photomicrographs of HE and NADPH diaphorase staining of the aorta from ZDF rats. Animals were anesthetized with pentobarbital and perfused with 4% paraformaldehyde in 0.1 mol/l phosphate buffer (pH 7.4) via the left ventricle, and aortic tissue was removed for HE and NADPH diaphorase staining as described in the text. (A) HE staining of the aorta from control rats; (B) HE staining of the aorta from insulin-treated rats; (C) HE staining of the aorta from insulin and L-NAME-treated rats; (D) NADPH diaphorase staining of the aorta from control rats; (E) NADPH diaphorase staining of the aorta from insulin-treated rats; (F) NADPH diaphorase staining of the aorta from insulin and L-NAME-treated rats. The scale bar represents 50 μ m.

ZDF rats, hypertension invariably occurs at a certain age after the onset of DM. This may be explained by proliferation of vascular smooth muscle induced by sympathetic stimulation (38) or insulin (6, 11, 39), or by reduction of insulin-induced vasodilatation due to increased insulin resistance (6, 11, 40–42). Therefore, it is considered that the ZDF rat may be a model of NIDDM associated with major vascular complications.

Systolic blood pressure was higher in the ZDF rat than in the Lean rat at the age of 12 weeks, but improved after insulin treatment in the ZDF rat. The antihypertensive effect of insulin continued until the end of the experiments. In contrast, administration of L-NAME, an inhibitor of NOS, together with insulin increased blood pressure above the level of non-treated ZDF rats. Although insulin is reported to stimulate reabsorption of sodium at the renal tubules (43), there was no significant difference in plasma osmolality and sodium concentrations among the three groups examined (control, insulin-treated and insulin and L-NAME-treated rats) (data not shown). NO_3^- was the predominant NO metabolite detected in rat plasma and the plasma level of NO_2^- was almost negligible in all the groups (data not shown). Plasma NOx ($\text{NO}_2^- + \text{NO}_3^-$) concentrations increased in the insulin-treated rats but not in either the non-treated control rats or the rats treated with insulin and L-NAME. In the rats treated with insulin and L-NAME, plasma NOx concentrations decreased. These results suggest that the antihypertensive effect of insulin is possibly mediated by NO. Systolic blood pressure increased in the insulin and L-NAME-treated rats above that of the control rats, and plasma NOx concentrations decreased in these animals below those of the control rats. These findings suggest that NO is acting tonically on the blood vessels in the ZDF rats. L-NAME is considered to have no effect on insulin secretion (44, 45), and the dose of insulin used in the present study is considerably higher than physiological plasma insulin concentrations (46). Therefore, an involvement of endogenous insulin may be negligible in the insulin-treated group. The physiological role of endogenous insulin on blood pressure remains to be clarified.

NO is considered to be a modulator of vascular tension, which is predominantly derived from vascular endothelial cells (47–49). To investigate the effect of exogenous insulin on NO production in the endothelial cells, we carried out *in vitro* NOS activity assay and NADPH diaphorase staining in the aorta tissues. NOS activity in the aorta was increased after insulin treatment, suggesting an increase in NOS molecule in the aorta by insulin administration. NOS activity of the aorta was not changed in the rats treated with insulin and L-NAME although plasma NOx concentration, as an index of *in vivo* NOS activity, was decreased compared with the control group.

HE staining showed that the insulin-treated aorta was dilated whereas the aorta treated with insulin and L-NAME was constricted. These results were compatible

with the blood pressure changes observed in the insulin-treated rats and insulin and L-NAME-treated rats. NADPH diaphorase staining revealed that the aorta of the insulin-treated rats was densely stained and that the aorta of the rats treated with insulin and L-NAME was stained even more densely than the insulin-treated rats. These results suggest that NOS molecule was induced in both the insulin-treated group and the insulin and L-NAME-treated group. Plasma NOx concentrations were higher in the insulin-treated rats than in the control rats. The data of the insulin-treated rats taken together suggest that insulin stimulates the synthesis of NOS molecule, resulting in an increase in NO production.

In the insulin and L-NAME-treated rats, *in vitro* NOS activity in the aorta and NADPH diaphorase staining are not consistent. The data of the rats treated with insulin and L-NAME apparently showed that *in vitro* NOS activity in the aorta was not increased but that NOS molecule detected with NADPH diaphorase staining was increased. Since plasma NOx concentrations were lower in the rats treated with insulin and L-NAME than in the control rats, *in vivo* NOS activity was decreased in the rats treated with insulin and L-NAME. It is assumed that reduction of NO production positively stimulates the synthesis of NOS molecule, resulting in dense staining of NADPH diaphorase in the endothelial cells of the insulin and L-NAME-treated rats. When *in vitro* NOS activity in the aorta of the insulin and L-NAME-treated rats was measured, L-NAME may still have bound to the NOS molecule, resulting in a discrepancy between NADPH diaphorase staining and *in vitro* NOS activity in the aorta of insulin and L-NAME-treated rats.

Although we did not examine the effect of insulin on small arteries, resistance of which is physiologically involved in regulating blood pressure, our findings in the aorta suggest that insulin treatment decreases blood pressure in the ZDF rats by enhancement of NO production via induction of NOS molecule synthesis in endothelial cells.

Acknowledgements

This work was supported in part by grants from the Ministry of Education and Culture, Japan, the Ministry of Health and Welfare, Japan, and the Shimane Institute for Intractable Diseases. We thank Mrs A Kanayama for her technical assistance and Mrs A Kawakami for her secretarial help.

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Received 30 December 1998

Accepted 19 January 1999