EXPERIMENTAL STUDY

Effect of insulin on nitric oxide synthase-like immunostaining of arteries in various organs in Zucker diabetic fatty rats

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Abstract

Objective: Recently we reported that insulin treatment improved hypertension by inducing nitric oxide synthase (NOS) in Zucker diabetic fatty (ZDF) rats. In the present study, we investigated subtypes of NOS induced by insulin in arteries in various organs of ZDF rats using immunohistochemistry.

Design: Following treatment with insulin, localization of two subtypes of NOS in the arterial tissues of various organs was identified.

Methods: Following 4 weeks of s.c. injection of insulin, the aorta, cerebral cortex, pancreas and kidney were stained with polyclonal anti-endothelial NOS (eNOS) or anti-inducible NOS (iNOS) antibodies.

Results: In the aortic tissue, eNOS-like immunostaining was observed equally in the insulin-treated group and the control group, whereas iNOS-like immunostaining was present more densely in the insulin-treated group. In the cerebral artery, eNOS-like immunostaining was observed in the endothelium and was enhanced in the insulin-treated group. In the control group, iNOS-like immunostaining was absent in the cerebral artery, whereas immunostaining was densely observed in the insulin-treated group. In the interlobular artery of the pancreas, both eNOS-like and iNOS-like immunostaining was present in the control group and was enhanced in the insulin-treated group. In kidney, both eNOS-like and iNOS-like immunostaining was more densely present in the arterial tissue of the insulin-treated group.

Conclusions: These results taken together suggest that insulin treatment induced NOS in arteries in various organs and that iNOS was more strongly induced than eNOS by insulin treatment in the ZDF rat.

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Introduction

Recently we have reported that insulin treatment improved hypertension in Zucker diabetic fatty (ZDF) rats (1, 2), a model of type 2 diabetes mellitus (3). 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 (4–11). Several lines of evidence suggest that insulin stimulates nitric oxide (NO) generation in endothelial cells (12–15). We observed that serum levels of NO metabolites were increased by insulin treatment in the ZDF rat (1, 2). Furthermore, NO synthase (NOS) activity and NADPH diaphorase staining were enhanced by insulin treatment (2). These data suggest that insulin treatment induces NOS in the ZDF rat. However, NADPH diaphorase staining does not always coincide with the presence of NOS (16). In the present study, we further investigated the effect of insulin on induction of NOS in arteries of various organs in the ZDF rat using

polyclonal antibodies against endothelial NOS (eNOS) and inducible NOS (iNOS).

Materials and methods

Antibodies and chemicals

Rabbit polyclonal anti-bovine eNOS antibody (anti-eNOS ab; B14553) was purchased from Calbiochem-Novabiochem (San Diego, CA, USA). Rabbit polyclonal anti-mouse iNOS antibody (anti-iNOS ab; PA3-030) was purchased from Affinity BioReagents (Golden, CO, USA). Both antibodies showed no cross-reactivity with other subtypes of NOS. All other chemicals were of the purest grade available from regular commercial sources.

Animals and insulin treatment

Eight male ZDF rats were obtained at the age of 7 weeks from Genetic Models Inc. (Indianapolis, IN, USA),

Table 1 Summary of the effect of insulin on immunostaining of eNOS and iNOS in various tissues on ZDF rats.

Tissues	NOS isozymes	Control	Insulin
Aorta			
	Endothelium	eNOS ++	++
		iNOS ++	++
	Smooth muscle	eNOS ±	±
		iNOS ±	++
Cerebral artery			
	Endothelium	eNOS ±	++
		iNOS -	+
	Smooth muscle	eNOS -	-
		iNOS -	++
Pancreas			
	Interlobular artery	eNOS ±	++
		iNOS +	++
	Exocrine cells	eNOS -	-
		iNOS -	±
Kidney			
	Arterial endothelium	eNOS ±	++
		iNOS -	±
	Arterial smooth muscle	eNOS -	+
		iNOS +	++
	Collecting tubule	eNOS ++	++
		iNOS +	+

-, not stained; ±, slightly stained; +, moderately stained; ++, densely stained.

housed in pairs, and kept in artificial lighting (lights on 0800 h, off 2000 h). Food and tap water were freely given until the age of 12 weeks. The rats were randomly divided into two groups, the insulin-treated group and the control group. Insulin therapy was started at the age of 12 weeks when plasma glucose levels were over 200 mg/ml. Insulin was injected s.c. in a dose of 25 U/kg per day between 1700 and 1800 h at 24 h intervals during the subsequent 4 weeks as previously described (17). In the control group, saline solution was injected s.c. in a dose of 1.25 ml/kg per day. During the experimental period, food and water intake as well as body weight were monitored at 1800 h every day.

Anti-NOS immunostaining

After treatment for 4 weeks, rats were anesthetized i.p. with sodium pentobarbital (50 mg/kg) and perfused with 300 ml 0.9% saline followed by 600 ml 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) via the left ventricle. The whole aorta, defined by the aortic arch through the bifurcation of the iliac artery, whole brain and kidney

Figure 1 Photomicrographs of NOS-like immunostaining of the aorta from ZDF rats. (A) eNOS-like immunostaining in control rats. (B) eNOS-like immunostaining in insulin-treated rats. (C) iNOS-like immunostaining in control rats. (D) iNOS-like immunostaining in insulin-treated rats. Solid arrows indicate endothelial cells, open arrows smooth muscle cells. Scale bar represents 20 μm. Representative sections are shown. Similar results were obtained from all rats examined.

were removed and immersed in the same fixative at 4 °C overnight. The pancreases was removed from each decapitated rat without perfusion and immersed in the same fixative as the other organs at 4 °C overnight. The fixed tissues were washed with water for 3 h and dehydrated in graded ethanol (70, 90, 99 and 100%) and chloroform, and embedded in 10% paraformaldehyde. The paraffin-embedded tissues were cut into 5 µm thick serial sections and placed on poly-L-lysine-coated glass slides. The paraffin-embedded serial sections were deparaffinized in 100% xylene, then rehydrated in graded ethanol with a step-wise decrease in concentration (99, 95, 90, 80 and 70%) and water. After microwave treatment in 10 mM citrate buffer for 5 min twice, the sections were incubated in 0.3% H₂O₂ in 100% methanol for 10 min at room temperature to block endogenous peroxidase. The sections were washed in PBS and non-specific binding was blocked by 30 min incubation in 1% normal goat serum in PBS at room temperature, and then the sections were incubated with anti-eNOS ab or anti-iNOS ab at 4 °C overnight. Anti-eNOS ab and anti-iNOS ab were diluted in PBS at 1:1000 and 1:750

respectively. For blank staining of eNOS and iNOS staining, the sections were incubated at 4 °C overnight with normal rabbit IgG fraction (K0113; Vector Laboratories, Burlingame, CA, USA) diluted in PBS to the same IgG concentrations as each antibody. After washing with PBS, the sections were incubated for 30 min at room temperature with biotinylated goat anti-rabbit immunoglobulins (Dako, Glostrup, Denmark) diluted in PBS at 1:600. For visualization, the sections were incubated with streptavidin-biotinylated horse-radish peroxidase (StreptABCComplex/HRP; Dako) for 40 min at room temperature and developed with diaminobenzidine. All the specimens compared were stained in the same experiment.

Ten independent sections of each rat were analyzed by two independent readers who were blinded for the treatment of the specimens. Positive NOS-like immunostaining was graded into three classes compared with blank staining: slightly stained if less than 20% of the examined cells were positively stained, moderately stained if 20–60% of the examined cells were positively stained and densely stained if more than 60% of the examined cells were positively stained.

Figure 2 Photomicrographs of NOS-like immunostaining of the cerebral artery from ZDF rats. (A) eNOS-like immunostaining in control rats. (B) eNOS-like immunostaining in insulin-treated rats. (C) iNOS-like immunostaining in control rats. (D) iNOS-like immunostaining in insulin-treated rats. Solid arrows indicate endothelial cells, open arrows smooth muscle cells. Scale bar represents 20 µm. Representative sections are shown. Similar results were obtained from all rats examined.

Results

To investigate the mechanism of antihypertensive effect of insulin, we studied immunostaining of NOS exclusively in arteries of examined organs. Since blank staining for eNOS and iNOS staining was negligible, positive cells for eNOS-like and iNOS-like immunostaining were definitely identified in all the sections of ZDF rats. The results of NOS-like immunostaining in the arteries of the examined organs are summarized in Table 1. In the ZDF rat, insulin treatment enhanced staining of NOS, especially iNOS, in the arteries of all the examined organs.

Aorta

In the control group, the endothelium was densely stained by anti-eNOS ab and the smooth muscle was slightly stained (Fig. 1A). The eNOS-like immunostaining was not changed in the insulin-treated group (Fig. 1B). The iNOS-like immunostaining was densely detected in the endothelium and slightly in the smooth

muscle of the control group (Fig. 1C). Insulin treatment enhanced iNOS-like immunostaining of the smooth muscle but not the endothelial cells (Fig. 1D).

Cerebral artery

In the control group, eNOS-like immunostaining was slightly identified in the endothelium (Fig. 2A). In the insulin-treated group, eNOS-like immunostaining of the endothelium was enhanced (Fig. 2B). In both groups, eNOS-like immunostaining was not detected in smooth muscle. In the control group, iNOS-like immunostaining was not observed in either the endothelium or smooth muscle (Fig. 2C). In the insulin-treated group, iNOS-like immunostaining of the cerebral artery was significantly detected in both the endothelium and the smooth muscle (Fig. 2D).

Pancreas

In the interlobular artery, eNOS-like immunostaining was slightly identified in the control group (Fig. 3A). In

Figure 3 Photomicrographs of NOS-like immunostaining of the pancreas from ZDF rats. (A) eNOS-like immunostaining of interlobular artery from control rats. (B) eNOS-like immunostaining of interlobular artery from insulin-treated rats. (C) iNOS-like immunostaining of interlobular artery from control rats. (D) iNOS-like immunostaining of interlobular artery and endocrine cells from insulin-treated rats. Arrows indicate arterial cells, arrow-heads endocrine cells. Scale bar represents 20 μ m. Representative sections are shown. Similar results were obtained from all rats examined.

the insulin-treated group, eNOS-like immunostaining of the interlobular artery was enhanced (Fig. 3B). Dense iNOS-like immunostaining was observed in the interlobular artery of both groups (Fig. 3C and D). In both groups, eNOS-like immunostaining was not detected in the endocrine cells (Fig. 3A and B). In the control group, iNOS-like immunostaining of the endocrine cells was absent (Fig. 3C), whereas it was densely detected in the insulin-treated group (Fig. 3D).

Kidney

In the control group, eNOS-like immunostaining was slightly detected in the arterial endothelium but not in the smooth muscle (Fig. 4A). Treatment with insulin enhanced eNOS-like immunostaining of both the arterial endothelium and the smooth muscle (Fig. 4B). In the control group, iNOS-like immunostaining was absent in the endothelium but moderately detected in the smooth muscle (Fig. 4C). In the insulin-treated group, iNOS-like immunostaining was slightly detected in the endothelium and densely in the smooth muscle

(Fig. 4D). The collecting tubule was densely positive for eNOS-like immunostaining both in the control group (Fig. 4A) and in the insulin-treated group (Fig. 4B). In the collecting tubules, iNOS-like immunostaining was moderately detected in both groups.

Discussion

In the control group, eNOS-like immunostaining was identified only in endothelium of the arteries, whereas iNOS-like immunostaining was observed in both endothelium and smooth muscle of arteries, as previously reported (16, 18–20). The present study demonstrates that insulin treatment enhanced eNOS-like immunostaining in endothelium and iNOS-like immunostaining in endothelium and smooth muscle of arteries in the various organs in the ZDF rat. These results were consistent with previous finding of a study with cultured human endothelial cells and smooth muscle cells (21). However, Kuboki *et al.* (22) reported that insulin treatment did not induce eNOS mRNA in microvessels taken from ZDF rats. The discrepancy may be due to difference in experimental conditions. In

Figure 4 Photomicrographs of NOS-like immunostaining of the kidney from ZDF rats. (A) eNOS-like immunostaining of artery and collecting tubules from control rats. (B) eNOS-like immunostaining of artery and collecting tubules from insulin-treated rats. (C) iNOS-like immunostaining of artery from control rats. (D) iNOS-like immunostaining of artery from insulin-treated rats. Solid arrows indicate endothelial cells, open arrows smooth muscle cells. Arrow-heads indicate collecting tubules. Representative sections are shown. Similar results were obtained from all rats examined.

contrast to our *in vivo* experiment, they studied the effect of insulin under *in vitro* conditions. Moreover, in the present study, insulin was administered for 4 weeks, whereas in their study, microvessels were incubated with insulin for 6 h. In the kidney, eNOS was densely stained in arterial smooth muscle of the insulin-treated group. This exceptional finding in the present study remains to be explained.

Insulin-induced NOS-like immunostaining in the arteries supported our previous observation that insulin-treatment induced NOS in aorta of ZDF rats as studied by NADPH diaphorase staining (2). The present data indicate that improvement of hypertension by insulin treatment was due to induction of NOS in the arteries in ZDF rats. In ZDF rats, insulin-induced NOS-like immunostaining was predominantly iNOS-like immunostaining. It is possible that insulin treatment stimulated NO production through iNOS induction in ZDF rats. Incubation of microvessels of non-diabetic rats with insulin for 6 h induced eNOS mRNA (22). However, it remains to be clarified whether chronic insulin treatment induces NOS under non-diabetic conditions. The present study suggests that insulin treatment improved general blood flow by inducing NOS in the arteries in the ZDF rat. It is speculated that NOS induction in the renal arteries improves renal blood flow and dysfunction of the renin-angiotensin system under hypertensive condition (23, 24). Although we did not examine the renin-angiotensin system in the ZDF rat, it is possible that insulin improves hypertension partly by reducing resistance of renal arterioles and serum renin level in these rats. The present data suggest that insulin treatment improves cerebral blood flow in the ZDF rat. It is assumed that central control of blood pressure was modulated by insulin treatment in the ZDF rat. In addition to arteries, insulin treatment induced NOS in pancreatic exocrine cells and renal tubules. The effects of insulin-induced NOS on pancreatic functions and renal functions remain to be investigated.

The present study suggests that in the ZDF rat insulin treatment affects various physiological conditions including glucose metabolism and blood pressure partly by induction of NOS.

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