ELSEVIER

European Journal of Pharmacology 394 (2000) 295–299

ejp

www.elsevier.nl/locate/ejphar

Evaluation of endothelial free radical release by vascular tension responses in insulin-resistant rat aorta

Kazuya Shinozaki [a], Tomio Okamura [b,*], Yoshihiko Nishio [a], Atsunori Kashiwagi [a], Ryuichi Kikkawa [a], Noboru Toda [b]

[a] *Third Department of Medicine, Shiga University of Medical Science, Seta, Otsu, Shiga 520-2192, Japan*
[b] *Department of Pharmacology, Shiga University of Medical Science, Seta, Otsu, Shiga 520-2192, Japan*

Received 18 January 2000; received in revised form 15 February 2000; accepted 18 February 2000

Abstract

Mechanical responses to superoxide anion scavengers and nitric oxide (NO) synthase inhibitors in aortic endothelial cells were compared in normal chow-fed rats and those made insulin-resistant by feeding of fructose. Cu^{2+}, Zn^{2+}-superoxide dismutase-induced vascular relaxation and superoxide production, measured by the lucigenin-enhanced chemiluminescence method, were greater in aortas from fructose-fed rats than in those from normal chow-fed rats. N^G-nitro-L-arginine-induced contractions due to suppression of NO synthase activity were smaller in aortas from fructose-fed rats. Vascular mechanical responses may reflect the generation of superoxide and NO by the endothelium. Thus, isometric tension studies may be a useful tool for evaluating the production of these radicals in blood vessels.

Keywords: Insulin resistance; Superoxide; Nitric oxide (NO); Isometric tension study

1. Introduction

Our recent study in animal models of insulin resistance showed an increased degradation of nitric oxide (NO) and endothelial dysfunction in response to superoxide anion radical (O_2^-) in vascular tissue (Shinozaki et al., 1999). Experiments on blood vessels from hypertensive (Nakazono et al., 1991) and hypercholesterolemic animals (Ohara et al., 1993) also suggest that an excess generation of O_2^- rapidly destroys NO and promotes the production of other active oxygen species, such as hydrogen peroxide (H_2O_2) and hydroxyl radical ($\cdot OH$), which are responsible for lipid peroxidation and damage of cellular membranes (Kontos and Kontos, 1995). However, from an analytical point of view, the release of O_2^- and NO in biological systems is not easily detected, because these radicals are quite labile and decompose each other (Archer, 1993). Therefore, in the present study, we aimed to estimate the basal release of endothelium-derived O_2^- and NO by measuring the superoxide dismutase-induced vasorelaxation and N^G-nitro-L-arginine (L-NA)-induced vasocontraction, respectively, and to correlate these data with biochemically measured O_2^- and NO production, the latter of which has been presented in an earlier study (Shinozaki et al., 1999).

2. Materials and methods

2.1. Animals

All protocols were approved by local institutional guidelines for animal care of Shiga University of Medical Science. Male Sprague–Dawley rats (Japan SLC, Shizuoka, Japan) weighing 150 g were divided into three groups and fed ad libitum on one of the following diets for 4 weeks: (1) a normal chow (''chow-fed''), (2) a normal chow with insulin infusion (''insulin-treated''), or (3) a diet high in fructose (''fructose-fed''). For continuous delivery of insulin, an incision was made in the midscapular region and a piece of insulin pellet (Linshin Canada, Ontario, Canada) was implanted (release rate 1.0 U/day) in the back of the rats. The normal chow (ORIENTAL YEAST, Tokyo, Japan) consisted of 58% carbohydrate (no fructose), 12%

* Corresponding author. Tel.: +81-77-548-2181; fax: +81-77-548-2183.
E-mail address: okamura@belle.shiga-med.ac.jp (T. Okamura).

0014-2999/00/$ - see front matter
PII: S0014-2999(00)00154-0

fat, and 30% protein (percentage of calories). The high fructose-diet (ORIENTAL YEAST) contained 67% carbohydrate (98% of which was fructose), 13% fat, 20% protein (percentage of calories). Although one may care that this different composition of two kind of chows may affect the experimental results, three groups had similar food intake, and the body weight and serum protein levels (chow-fed rats, 5.8 ± 0.2 g/dl; insulin-treated rats, 6.1 ± 0.3 g/dl; fructose-fed rats, 5.9 ± 0.3 g/dl) of the rats after 4 weeks were similar in all groups, as previously reported (Zavaroni et al., 1982). Blood pressure was measured the day before the experiment by means of an electrosphymgomanometer placed on the tail of the rat, which had been pre-warmed for 15 min. Insulin sensitivity was measured by the steady-state plasma glucose method (Harano et al., 1981). Plasma glucose concentrations were measured by the standard enzymatic method and insulin by radioimmunoassay using anti-rat insulin antibody.

2.2. Isometric tension studies

The thoracic aorta (0.6–0.8 cm outside diameter) was cut into segments with special care being taken to preserve the endothelium, and specimens were cut open and suspended in organ chambers containing (mmol/l) a modified Ringer–Locke solution (NaCl 120, $CaCl_2$ 2.2, $MgCl_2$ 1.0, $NaHCO_3$ 25.0, and dextrose 5.6 (pH 7.4)) as previously described (Shinozaki et al., 1999). To prevent the synthesis of prostaglandins, the strips were treated with 10 μmol/l indomethacin and partially precontracted with L-phenylephrine ($1–3 \times 10^{-7}$ mol/l). After a plateau was attained, the strips were exposed to acetylcholine ($10^{-9}–10^{-5}$ mol/l) to construct dose–response curves. At the end of each experiment, 100 μmol/l papaverine (Dainippon, Osaka, Japan) was added to induce maximal relaxation, which was taken as 100% for relaxation induced by agonists. In some strips, the endothelium was removed by gently rubbing the intimal surface with a cotton ball. Endothelium removal was verified by abolition or marked suppression of the relaxations caused by 1 μmol/l acetylcholine.

2.3. Measurement of ex vivo aortic O_2^- production

O_2^- production in aortic segments (20 mm) was measured using the lucigenin-enhanced chemiluminescence method (Ohara et al., 1993; Li et al., 1998). The segments were exposed to modified Krebs/HEPES buffer (pH7.4) and allowed to equilibrate for 30 min at 37°C. After 5 min of dark adaptation, scintillation vials containing 2 ml Krebs/HEPES buffer with 50 μmol/l lucigenin were placed into a scintillation counter (TRI-CARB1500, PACKARD Instrument, Meriden, CT) switched to the ''out of coincidence'' mode. Lucigenin counts are expressed as cpm/mg of dry weight of vessel. Background counts were determined from vessel-free incubations and subtracted from the readings obtained using vessels.

2.4. Measurement of vascular O_2^- scavenging activity

Vascular superoxide-scavenging activity was spectrophotometrically measured by a modification of the method originally described by Salin and McCord (1974). Segments of thoracic aorta were harvested as described above and homogenized in phosphate-buffered saline (2 ml, pH 7.4) using a homogenizer, and the homogenates were centrifuged at $13,600 \times g$ for 15 min. The aliquot (0.1 ml) was incubated with 100 μmol/l xanthine, 15 μmol/l cytochrome *c* (horse heart type IV), 20 mmol/l $NaHCO_3$, 1 mmol/l NaN_3, and 0.1 mmol/l EDTA. The assay was initiated by the addition of 0.025 ml xanthine oxidase (1.0 U/ml) and the resultant superoxide generation was estimated by the increase in absorbance at 550 nm over the 20 s of the reaction. The residual superoxide dismutase-independent superoxide-scavenging activity was determined from identically prepared vessels that had been incubated with 10 mmol/l diethyldithiocarbamate (an inhibitor of Cu^{2+}, Zn^{2+}-superoxide dismutase) for 30 min.

2.5. Measurement of NO synthase activity in aortic endothelial cell

Endothelial NO synthase activity was measured by the conversion of [^{3}H]L-arginine (New England Nuclear Research Products, Boston, MA., USA) to [^{3}H]L-citrulline as previously described (Rees et al., 1996). The Ca^{2+}-dependent enzyme activity of endothelial NO synthase was determined as the difference between the [^{3}H]L-citrulline generated from control samples without EGTA and those containing 3 mmol/l EGTA. Values were corrected for the amount of protein present.

3. Results

As shown in Table 1, animals receiving insulin infusion demonstrated a significant ($P < 0.05$) decrease in plasma glucose levels (77.4 ± 4.9 mg/dl) when compared to either chow-fed (102 ± 7.2) or fructose-fed (97.8 ± 1.9) rats. Both insulin-treated (59.6 ± 5.1 μU/ml) and fructose-fed (30.0 ± 1.4) rats showed increased ($P < 0.001$) plasma insulin levels compared with chow-fed rats (17.0 ± 1.2). Fructose-fed rats (141 ± 3 / 84 ± 3 mm Hg) showed a significant ($P < 0.01$) elevation of blood pressure (systolic/diastolic) compared with other two groups (chow-fed rat; $116 \pm 2/71 \pm 3$, Insulin-treated rats; $114 \pm 2/74 \pm 5$). Furthermore, fructose-fed rats (192 ± 15 mg/dl) exhibited a significant ($P < 0.01$) increase in steady-state plasma glucose level when compared with the other groups (chow-fed rats; 126 ± 7, insulin-treated rats; 122 ± 8). These results clearly indicated that the rats fed on high-fructose diets had acquired insulin resistance, whereas the rats with chronic exogenous hyperinsulinemia showed normal insulin sensitivity.

Table 1
Metabolic characteristics and blood pressure of the rats

	Chow-fed	Insulin-treated	Fructose-fed
Weight (g)	281 ± 16	279 ± 9	302 ± 5
Glucose (mg/dl)	102 ± 7.2	77.4 ± 4.9[a]	97.8 ± 1.9[b]
Insulin (μU/ml)	17.0 ± 1.2	59.6 ± 5.1[c]	30.0 ± 1.4[c,d]
Total cholesterol (mg/dl)	66.3 ± 3.7	65.8 ± 3.1	79.2 ± 2.5[a,e]
Triglyceride (mg/dl)	133 ± 19.0	67.4 ± 8.6	309 ± 36.8[c,d]
Systolic blood pressure (mm Hg)	115.9 ± 2.1	114.6 ± 2.0	141.4 ± 1.8[c,d]
Diastolic blood pressure (mm Hg)	71.3 ± 3.6	74.4 ± 4.8	84.1 ± 1.5[a,b]

Data are expressed as means ± S.E.M. Comparisons among those groups for metabolic data and blood pressure were performed using ANOVA with a post-hoc Scheffe's comparison.
[a] $P < 0.01$ vs. chow-fed rats.
[b] $P < 0.05$ vs. insulin-treated rats.
[c] $P < 0.001$ vs. chow-fed rats.
[d] $P < 0.001$ vs. insulin-treated rats.
[e] $P < 0.01$ vs. insulin-treated rats.

The contractile responses to 30 mmol/l KCl of aortic strips from chow-fed, insulin-treated, and fructose-fed rats were 1.07 ± 0.07, 1.12 ± 0.06, and 1.01 ± 0.05 g, respectively ($n = 7$, $P < 0.05$). As shown in Fig. 1A, the magnitude of Cu^{2+}, Zn^{2+}-superoxide dismutase-induced vascular relaxation in aortic strips from insulin-treated rat and fructose-fed rats was significantly greater than that in aortic strips from the chow-fed rats. L-NA-induced contractions, estimated relative to the KCl-induced contraction, were significantly greater in the aortic strips from insulin-treated rats than in aortic strips from chow-fed and fructose-fed rats (Fig. 1B). However, neither superoxide dismutase nor L-NA altered the arterial tone in the strips without the endothelium (data not shown, $n = 5$). The endothelium-intact aortic strips from fructose-fed rats had an impaired relaxation in response to either acetylcholine or calcium ionophore A23187, whereas such an impairment is not found in aortic strips from insulin-treated rats (Shinozaki et al., 1999). Treatment with 0.1 mmol/l L-NA and removal of the endothelium abolished the acetylcholine-induced relaxation in aortic strips obtained from the three different groups, suggesting the involvement of NO liberated from the endothelium.

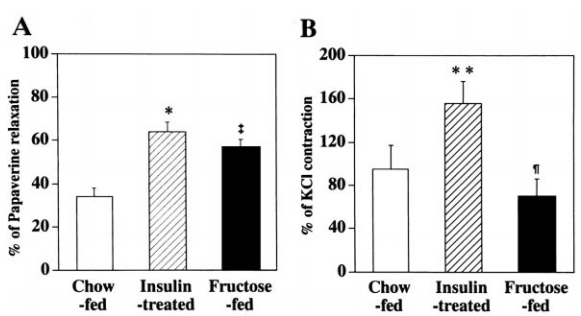

Fig. 1. Effects of superoxide dismutase (200 U/ml) (A) and L-NA (10^{-4} mol/l) (B) on either vascular contraction or relaxation of aortic strips with the endothelium. Panel A: Relaxation in response to superoxide dismutase was studied after the vessels had been pre-constricted with L-phenylephrine, and the magnitude of relaxation is expressed as a percentage of the maximal relaxation induced by 0.1 mmol/l papaverine. Panel B: Contraction in response to L-NA was studied after the vessels had been pre-constricted with L-phenylephrine, and the magnitude of contraction is expressed as a percentage of the contraction induced by 30 mmol/l KCl. All experiments were performed in the presence of indomethacin (10^{-5} mol/l). The results are expressed as means ± S.E.M. of five different experiments. $^{*}P < 0.0001$, ‡$P < 0.001$, $^{**}P < 0.05$ vs. chow-fed rats. ¶$P < 0.01$ vs. insulin-treated rats.

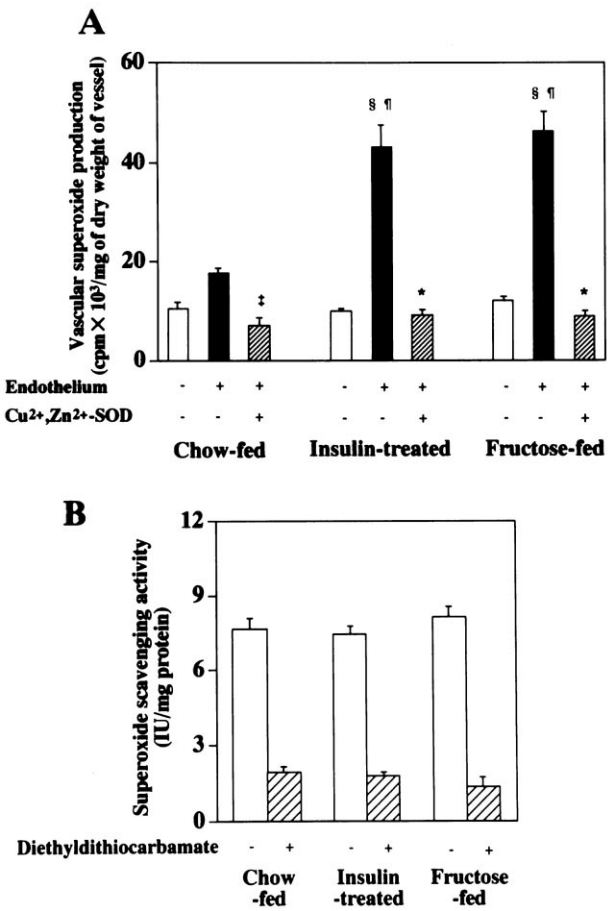

Fig. 2. Panel A: Comparison of vascular superoxide anion production in the absence (open columns) or presence (closed columns) of endothelium in aortas from chow-fed ($n = 9$), insulin-treated ($n = 9$), and fructose-fed ($n = 9$) rats. The vessels from each group were pre-exposed for 30 min to 200 U/ml superoxide dismutase (SOD) (hatched columns). Panel B: Comparison of vascular superoxide-scavenging activity among the three groups. The enzyme activity was calculated from a linear dose–response curve obtained using 0.1–10 U/ml bovine erythrocyte Cu^{2+}, Zn^{2+}-superoxide dismutase and expressed as units of superoxide dismutase normalized to the protein content. The residual Cu^{2+}, Zn^{2+}-superoxide dismutase-independent superoxide-scavenging activity was determined from identically prepared vessels that were incubated with 10 mmol/l diethyldithiocarbamate (an inhibitor of Cu^{2+}, Zn^{2+}-superoxide dismutase) for 30 min. Values are expressed as means ± S.E.M. §$P < 0.0001$ vs. each vessel without endothelium incubated in buffer alone. ¶$P < 0.0001$ vs. the corresponding control vessels with endothelium obtained from chow-fed rats. ‡$P < 0.01$, $^{*}P < 0.001$ vs. the corresponding control vessels with endothelium incubated in buffer alone, using Student's unpaired *t*-test. The data for the three groups were analyzed by multiple comparison test using ANOVA with a post-hoc Scheffe's comparison.

As shown in Fig. 2A, superoxide production by aortic segments from insulin-treated and fructose-fed rats was over two-fold higher than that of segments from chow-fed rats ($P < 0.0001$). Endothelial denudation did not significantly reduce O_2^- levels in vessels from chow-fed rats, while a marked reduction was seen in vessels from both insulin-treated and fructose-fed rats. Thus, after removal of the endothelium, the O_2^- production did not differ among the three groups. Furthermore, incubation of endothelium-intact segments from the three groups with 200 U/ml superoxide dismutase for 10 min markedly decreased the lucigenin signal.

As shown in Fig. 2B, the activity of Cu^{2+}, Zn^{2+}-superoxide dismutase, a major source of superoxide catabolism in arterial tissue, did not differ among the groups. After inhibition of Cu^{2+}, Zn^{2+}-superoxide dismutase activity with 10 mM diethyldithiocarbamate, the mean values of the residual water-soluble superoxide scavenging activity (less than 25% of total activity) were also not different among the three groups. In the absence of endothelium, the activity of Cu^{2+}, Zn^{2+}-superoxide dismutase and that of the other superoxide dismutase isoenzymes did not differ among the three groups (data not shown).

The NO synthase activity of the Ca^{2+}-dependent enzyme was significantly greater in aortic endothelial cells ($n = 4$) from insulin-treated rats (177.5 ± 12.0 pmol/min/mg protein) than it was in cells from either chow-fed (63.2 ± 8.7, $P < 0.0001$) or fructose-fed (24.3 ± 12.0, $P < 0.0001$) rats. The enzyme activity was significantly depressed in cells from fructose-fed rats compared with that of cells from chow-fed rats ($P < 0.05$). There was no significant Ca^{2+}-independent NO synthase activity in aortic endothelial cell homogenates from the three groups of rats.

4. Discussion

It is quite difficult to identify and quantify biologically generated free radicals in vascular tissues. The net vascular production of O_2^- is a function of both O_2^- generation and local O_2^- scavenging (Kontos and Kontos, 1995). Arterial tissues contain considerable amounts of superoxide dismutase, which effectively accelerates the dismutation of O_2^-. In the present study, the activity of intrinsic superoxide dismutase did not differ among aortas from different rat groups. The addition of Cu^{2+}, Zn^{2+}-superoxide dismutase to aortic strips that were precontracted by L-phenylephrine produced a significant relaxation of isolated rat aorta with endothelium, which is consistent with the results obtained with the lucigenin method. The removal of the endothelium abolished the response to superoxide dismutase. These findings suggest that the vasodilative effect of superoxide dismutase is caused by the protection of NO released from vascular endothelial cells under basal conditions. The addition of L-NA to aortic strips produced a significant contraction of isolated rat aortas which was abolished by removal of the endothelium. Our previous study has demonstrated that the L-NA-induced vascular contraction is associated with the basal release of endothelium-derived NO in aortic strips (Toda et al., 1993). In the present study, we found that the magnitude of the L-NA-induced vascular contraction was in parallel with the activity of endothelial NO synthase. These results suggest that the degree of endothelium-dependent vasorelaxation elicited by Cu^{2+}, Zn^{2+}-superoxide dismutase and vasoconstriction elicited by L-NA may partly reflect the amount of O_2^- and NO released from the endothelium, respectively. Because of the complex interactions among various free radical species (Fraile et al, 1994; Yang et al., 1999) released from the vascular wall, the radical species responsible for the superoxide dismutase-induced relaxation have not been identified. Whether it is NO or hydrogen peroxide remains to be clarified. A recent study has demonstrated that endothelial-dependent relaxation in insulin-resistant rats is impaired before the development of hypertension and does not significantly worsen after hypertension has developed (Katakam et al., 1999). These findings suggest that impaired endothelial function results in an increase in systemic vascular resistance and blood pressure. Isometric tension studies may be useful to corroborate direct measurements of free radical production in studies of isolated vessels.

Acknowledgements

This study was supported in part by a research grant-in-aid from the Japan Society for the Promotion of Science (JSPS) Fellows and the Ministry of Education, Science, and Culture of Japan.

References

Archer, S., 1993. Measurement of nitric oxide in biological models. FASEB. J. 7, 349–360.

Fraile, M.L., Conde, M.V., Sanz, L., Moreno, M.J., Marco, E.J., Lopez de Pablo, A.L., 1994. Different influence of superoxide anions and hydrogen peroxide on endothelial function of isolated cat cerebral and pulmonary arteries. Gen. Pharmacol. 25, 1197–1205.

Harano, Y., Ohgaku, S., Kosugi, K., Yasuda, H., Nakano, T., Kobayashi, M., Hidaka, H., Izumi, K., Kashiwagi, A., Shigeta, Y., 1981. Clinical significance of altered insulin sensitivity in diabetes mellitus assessed by glucose, insulin, and somatostatin infusion. J. Clin. Endocrinol. Metab. 52, 982–987.

Katakam, P.V.G., Ujhelyi, M.R., Hoenig, M.E., Miller, A.W., 1999. Endothelial dysfunction precedes hypertension in diet-induced insulin resistance. Am. J. Physiol. 275, R788–R792.

Kontos, H.A., Kontos, M.C., 1995. Role of products of univalent reduction of oxygen in hypertensive vascular injury. In: Laragh, J.H., Brenner, B.M. (Eds.), Hypertension: Pathophysiology, Diagnosis, and Management. Raven Press, New York, pp. 685–696.

Li, Y., Zhu, H., Kuppusamy, P., Roubaud, V., Zweier, J.L., Trush, M.A., 1998. Validation of lucigenin (Bis-*N*-methylacridinium) as a chemilu-

migenic probe for detecting superoxide anion radical production by enzymatic and cellular systems. J. Biol. Chem. 273, 2015–2023.

Nakazono, K., Watanabe, N., Matsuno, K., Sasaki, J., Sato, T., Inoue, M., 1991. Does superoxide underlie the pathogenesis of hypertension. Proc. Natl. Acad. Sci. U. S. A. 88, 10045–10048.

Ohara, Y., Peterson, T.E., Harrison, D.G., 1993. Hypercholesterolmia increases endothelial superoxide anion production. J. Clin. Invest. 91, 2546–2551.

Rees, D., Ben-Ishay, D., Moncada, S., 1996. Nitric oxide and the regulation of blood pressure in the hypertension-prone and hypertension-resistant sabra rat. Hypertension 28, 367–371.

Salin, M.L., McCord, J.M., 1974. Superoxide dismutase in polymorphonuclear leukocytes. J. Clin. Invest. 54, 1005–1009.

Shinozaki, K., Kashiwagi, A., Nishio, Y., Okamura, T., Yoshida, Y., Masada, M., Toda, N., Kikkawa, N., 1999. Abnormal biopterin metabolism is a major cause of impaired endothelium-dependent relaxation through NO/O_2^- imbalance in insulin-resistant rat aorta. Diabetes 48, 2437–2445.

Toda, N., Ayajiki, K., Okamura, T., 1993. Endothelial modulation of contractions caused by oxyhemoglobin and N^G-nitro-L-arginine in isolated dog and monkey cerebral arteries. Stroke 24, 1584–1589.

Yang, Z., Zhang, A., Altura, B.T., Altura, B.M., 1999. Hydrogen peroxide-induced endothelium-dependent relaxation of rat aorta involvement of Ca^{2+} and other cellular metabolites. Gen. Pharmacol. 33, 325–336.

Zavaroni, I., Chen, Y.-D.I., Reaven, G.M., 1982. Studies of the mechanism of fructose-induced hypertriglyceridemia in the rat. Metabolism 31, 1077–1083.