Effects of Chronic Administration of L-Arginine on Vasoactive Responses induced by Endothelin-1 and its Plasma Level in Streptozotocin-Induced Diabetic Rats

Ayako MAKINO1 and Katsuo KAMATA1

1Department of Physiology and Morphology, Institute of Medicinal Chemistry, Hoshi University, Shinagawa-ku, Tokyo 142-8501, Japan

Abstract

To investigate the mechanism underlying increased endothelin-1 (ET-1) release in diabetic rats, we administered L-arginine chronically to streptozotocin (STZ)-induced diabetic rats. The plasma concentrations of glucose, ET-1 and NOx (NO2– + NO3–) were all significantly raised at 10 weeks after the STZ injection. Chronic administration of L-arginine resulted in a significantly higher plasma NOx concentration and a significantly lower plasma ET-1 level at 10 weeks compared with the untreated diabetic group. ET-1 induced a biphasic vasodilator/vasoconstrictor response in the perfused isolated mesenteric arterial beds from all groups. The vasodilatation was significantly greater in diabetic rats than in age-matched controls. Chronic oral L-arginine administration had no significant effect on the enhanced ET-1-induced vasodilatation seen in the untreated diabetic rats. The vasoconstrictions induced by ET-1 and methoxamine were significantly attenuated in STZ-diabetic rats. The attenuated vasoconstrictor response to ET-1, but not that to methoxamine, was further attenuated by chronic treatment with L-arginine. We conclude that since chronic L-arginine administration not only reduced the increase in plasma ET-1 levels but also further attenuated the ET-1-induced vasoconstriction without affecting the change in vasodilatation, chronic L-arginine administration could be valuable for the treatment of the symptoms of diabetic mellitus related to ET-1.

Key words: endothelin-1, diabetes, L-arginine, nitric oxide, mesenteric artery, rat

Introduction

Nitric oxide (NO) and endothelin-1 (ET-1) are major endothelium-derived factors with opposing effects on the function and structure of the vessel wall. Recent evidence suggests a complex interaction of endothelium-derived factors, including ET-1 and NO, in the control of vascular smooth muscle tone in health and disease (Lucher et al., 1993). ET-1 is a 21-amino-acid peptide with potent vasoconstrictor actions in animals and humans (Yanagisawa et al., 1989;...
Haynes and Webb, 1994). NO, a short-lived radical derived from the amino acid l-arginine by NO synthase, has a direct vasodilator action (Palmer et al., 1988; Moncada et al., 1991). In vitro experiments have shown that an increase in the cyclic GMP level occurring as a consequence of increased levels of NO inhibits the synthesis of ET-1 (Boulanger and Lucher, 1990), ET-1-induced contraction (Lerman et al., 1992) and ET-1-induced inositol phosphate production (Millard et al., 1998). Furthermore, it has been reported that inhibition of NO synthesis in rats enhances the tonic pressure response to ET-1 and also results in an increased plasma level of ET-1 (Richard et al., 1995; Filep, 1997).

Current evidence suggests that ET-1 may be involved in several pathological states (Miyauchi and Goto, 1999). In pathophysiological conditions involving endothelial dysfunction, the expression of ET-1 is increased (Roux et al., 1999). Although alterations in the release and action of ET-1 occur in diabetes mellitus, the nature and direction of these changes is still controversial. We recently showed that in streptozotocin (STZ)-induced diabetic rats the plasma ET-1 level is elevated (Makino and Kamata, 1998a, 2000; Makino et al., 2001; Kanie and Kamata, 2002). In diabetic patients, both normal and impaired endothelium-dependent vasodilatation has been reported by workers using different techniques in different vascular beds have been reported (Saenz et al., 1989; Makimattila et al., 1997; Huvers et al., 1999; Calver et al., 1992; Elliott et al., 1993; Smits et al., 1993). Several studies support the concept that NO bioactivity is decreased in IDDM due to enhanced metabolism of NO resulting from increased superoxide synthesis (Cohen, 1993; Kobayashi and Kamata, 1999, 2001). Moreover, it has been reported that impaired endothelium-dependent vasodilatation can be restored by exogenous l-arginine in aortic rings obtained from STZ-induced diabetic rats (Pieper et al., 1995; Pieper and Peltier, 1995).

Thus, it is likely that enhanced metabolism of NO is related to the increase in the plasma ET-1 level seen in diabetes. To test this hypothesis, we investigated whether chronic treatment of STZ-induced diabetic rats with l-arginine, as a substrate for NO, would affect the increased release of ET-1 seen in such animals. In addition, we also examined whether the altered responsiveness to ET-1 seen in isolated mesenteric arterial beds from diabetic rats would be affected by the chronic administration of l-arginine. This is, to our knowledge, the first study concerning the effect of oral administration of l-arginine on ET-1 release in diabetic rats.

Methods

Materials

Streptozotocin, methoxamine hydrochloride, l-arginine, N\(^\text{G}\)-nitro-l-arginine (l-NOARG), papaverine hydrochloride and bovine serum albumin (Fraction V) were all purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Acetylcholine chloride was from Daiichi Pharmaceutical Co. (Tokyo, Japan) and endothelin-1 (ET-1) was from Peptide Institute, Inc. (Osaka, Japan).

Animals and induction of diabetes mellitus

This study was conducted in accordance with the Guide for the Care and Use of Laboratory
Animals adopted by the Committee on the Care and Use of Laboratory Animals of Hoshi University (which is accredited by the Ministry of Education, Science, Sports and Culture, Japan). Food and water were available ad libitum to all animals. Male Wistar rats, 8 weeks old and 220–250 g in weight, were divided randomly into 3 groups. Group 1 animals were injected with citrate buffer alone. Group 2 and group 3 animals each received a single injection via the tail vein of STZ 66 mg/kg, dissolved in citrate buffer. Group 3 animals subsequently received L-arginine in their drinking water (0.1%) for 4 weeks (starting 6 weeks after the injection of STZ). Blood pressure and heart rate were recorded by the tail-cuff method. The concentration of glucose in the plasma was determined by the O-toluidine method (Dubowski, 1962).

Radioimmunoassay of ET-1

Plasma samples taken 10 weeks after injection of STZ or buffer were extracted using Amprep C2 columns (Amersham International plc., Buckinghamshire, U.K.) following Amprep activation by 2 ml of 100% methanol followed by 2 ml water. One milliliter of each plasma sample was acidified with 0.25 ml 2 M HCl, centrifuged at 10000 g for 5 min at room temperature and then loaded onto the column. The column was washed with 5 ml of 0.1% trifluoroacetic acid (TFA). Immunoreactive-endothelin was eluted with 2 ml of 80% acetonitrile/water containing 0.1% TFA. Then, the eluate was dried down under nitrogen and the resulting pellet reconstituted using assay buffer (0.02 M borate buffer, pH 7.4, containing 0.1% sodium azide). The concentration of ET-1 in the eluate of each plasma sample was determined by radioimmunoassay using a commercially available kit (endothelin 1-21 specific [125I] assay system; Amersham International plc., Buckinghamshire, England).

Measurement of NO2– and NO3–

The concentration of nitrite and nitrate in the plasma was assayed by the method described elsewhere (Yamada et al., 1997). To 0.5 ml of each plasma sample 0.5 ml 100% methanol was added, and it was then centrifuged at 5000 g for 10 min at 4°C. Briefly, the NO2– and NO3– in the each supernatant were separated by means of a reverse-phase separation column packed with polystyrene polymer (NO-PAK, 4.6 × 50 mm, Eicom), then NO3– was reduced to NO2– in a reduction column packed with copper-plated cadmium filings (NO-RED; Eicom). The NO2– was mixed with a Griese reagent to form a purple azo dye in a reaction coil. The separation and reduction columns and the reaction coil were then placed in a column oven set at 35°C. The absorbance of the product dye at 540 nm was measured using a flow-through spectrophotometer (NOD-10, Eicom). The mobile phase, which was delivered by a pump at a rate of 0.33 ml/min, was 10% methanol containing 0.15 M NaCl/NH4Cl and 0.5 g/L 4 Na-EDTA. The Griese reagent, which was 1.25% HCl containing 5 g/L sulfanilamide with 0.25 g/L N-naphthylethylendiamine, was delivered at a rate of 0.1 ml/min. The concentration of NO2– and NO3– and the reliability of the reduction column were examined in each experiment.

Preparation of the mesenteric arterial bed for perfusion

Ten weeks after treatment with STZ or buffer, rats were anaesthetized with ether and then given an intravenous injection of 1000 units/kg of heparin. Following this injection, the
mesenteric arterial bed was rapidly dissected out and placed into modified Krebs-Henseleit solution (KHS, composition in mM: NaCl 118.0; KCl 4.7; NaHCO3 25.0; CaCl2 1.8; NaH2PO4 1.2; MgSO4 1.2; dextrose 11.0; 0.25% bovine serum albumin). The mesenteric artery and vein were tied off near the caecum, and the remaining intestine was separated from the arterial bed along the intestinal wall. The mesenteric arterial bed was then perfused using the method described previously by us (Kamata et al., 1996; Kamata and Makino, 1997; Makino and Kamata, 1998). Briefly, warm (37°C), oxygenated (95% O2–5% CO2) KHS was pumped into the mesenteric arterial bed, using a peristaltic pump operating at a rate of 5 ml/min, through a cannula inserted into the superior mesenteric artery. Vascular responses were detected as changes in perfusion pressure; this was monitored continuously by way of a pressure transducer (Nihon Kohden, Model AP2001, Tokyo, Japan) and recorded on a pen recorder.

In the first series of experiments, we examined the vasodilator responses of perfused mesenteric arterial beds to ET-1. Following a 60 min equilibration period, the perfusion circuit was transformed into a closed system by collecting the perfusate in a second bath and thence recirculating it through the mesenteric arterial bed. The total volume of the closed system was 50 ml, and agents were administered via the bath. In some preliminary experiments, the mesentery preparation was constricted by perfusion with a solution containing 4 × 10^{-6} to 3 × 10^{-5} M methoxamine, which resulted in an increase in perfusion pressure of approximately 105–120 mmHg, and then maximally relaxed with a perfusion solution containing 10^{-6} M ACh, a response which confirmed the integrity of the endothelium in our preparation. In order to standardize the vasodilator responses obtained with different drugs, papaverine (10^{-4} M) was injected into each mesentery at the end of experiment and the resulting vasodilator response expressed as 100%. Method for the ET-1-induced vasoconstriction (+vasoconstriction or −vasoconstriction) and vasodilatation were shown in Fig. 1. In each preparation, once the methoxamine-induced vasoconstriction had reached a plateau, vasodilator responses to ET-1 were elicited in a single concentration-effect manner.

In a second series of experiments, we examined the contractile responses to endothelin-1 and methoxamine, each in a cumulative manner. To investigate the influence of L-arginine (10^{-4} M), or L-NOARG (10^{-4} M) plus 10^{-5} M indomethacin on these agonist-induced responses, the mesentery was incubated in the appropriate solution for 30 min before the addition of methoxamine. Each preparation was used to test only agonist.

**Statistical analysis**

Data are expressed as the mean ± S.E.M. When appropriate, statistical differences were assessed by Dunnett’s test for multiple comparisons after a one-way analysis of variance (ANOVA). Statistical comparisons between concentration-response curves were made by means of a two-way ANOVA with Bonferroni’s correction performed post-hoc to correct for multiple comparisons. P<0.05 was considered significant in both types of test.
Results

General characteristics

Body weight was lower in STZ-induced diabetic rats, at 10 weeks after the STZ injection, than in the age-matched controls. It was even lower in chronic L-arginine-treated diabetic rats (Table 1). Haemodynamic variables and the plasma levels of glucose, ET-1 and NOx were measured at 10 weeks after the STZ injection (Table 1).

Both systolic blood pressure and heart rate were lower in STZ-induced diabetic rats than the age-matched controls. The concentrations of plasma glucose, ET-1 and NOx (NO2− + NO3−) were all significantly raised at 10 weeks after the injection. Chronic administration of L-arginine reduced both systolic blood pressure and heart rate to below the levels seen in untreated diabetic rats. The plasma glucose concentration was not different between L-arginine-treated and -untreated STZ-induced diabetic rats. Chronic L-arginine treatment resulted in a significantly higher plasma NOx concentration compared with control group and a significantly lower plasma ET-1 level at 10 weeks compared both with the control and untreated diabetic groups.

Vascular responses induced by ET-1

The basal perfusion pressures in mesenteric arterial beds from age-matched controls, STZ-induced diabetic rats and chronic L-arginine-treated diabetic rats were 58.9 ± 1.8 mmHg, n=6, 55.2 ± 0.7 mmHg, n=5, and 55.7 ± 0.9 mmHg, n=5, respectively (P>0.05). Perfusion with methoxamine (4 × 10−6 to 3 × 10−8 M) increased the perfusion pressure to 116.4 ± 4.7 mmHg, n=6, 101.0 ± 5.9 mmHg, n=5, and 100.1 ± 8.1, n=5, respectively. Therefore, the initial perfusion pressure was adjusted to 100–120 mmHg in subsequent experiments under various experimental conditions. ET-1 (10−8 M) induced a transient vasodilatation followed by a marked vasoconstriction in the mesenteric arterial bed preconstricted with methoxamine (Fig. 1). The maximum vasodilatation evoked by ET-1 in the STZ-induced diabetic rats was significantly larger than that evoked in the controls (Fig. 2). By contrast, the maximum vasoconstriction in STZ-induced diabetic rats was significantly smaller than that seen in the control group. Chronic L-arginine treatment had no effect on the vasodilator response to ET-1. By contrast, the maximum vasoconstriction in the chronic L-arginine-treated diabetic rats was almost abolished.

Table 1 Haemodynamic variables in control and L-arginine-treated or -untreated STZ-induced diabetic rats

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control (n=9)</th>
<th>Diabetic (n=9)</th>
<th>L-arginine-treated diabetic (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>501.7 ± 10.8</td>
<td>249.0 ± 10.6***</td>
<td>202.5 ± 9.5***##</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>149.7 ± 3.3</td>
<td>138.7 ± 2.9*</td>
<td>107.9 ± 1.9***###</td>
</tr>
<tr>
<td>Heart rate (beats/min)</td>
<td>374.0 ± 13.7</td>
<td>263.2 ± 12.2***</td>
<td>244.0 ± 2.9***###</td>
</tr>
<tr>
<td>Plasma glucose (mg/dl)</td>
<td>155.5 ± 3.4</td>
<td>637.2 ± 23.5***</td>
<td>638.9 ± 32.0***</td>
</tr>
<tr>
<td>Plasma ET-1 (pg/ml)</td>
<td>24.1 ± 1.8</td>
<td>30.4 ± 1.2*</td>
<td>9.7 ± 1.7***###</td>
</tr>
<tr>
<td>Plasma Nox (nmol/ml)</td>
<td>5.80 ± 0.27</td>
<td>7.43 ± 0.78*</td>
<td>8.15 ± 0.72**</td>
</tr>
</tbody>
</table>

Values are the mean ± S.E.M. *, P<0.05; **, P<0.01; ###, P<0.001 vs. controls; ##, P<0.01; ###, P<0.001 vs. STZ-induced diabetic rats.
compared to than that seen in the untreated diabetic rats (Figs. 2 & 3).

**Concentration-dependent vasoconstrictor response to ET-1 or methoxamine**

Infusion of cumulative concentrations of ET-1 (10^{-10} to 10^{-7} M) caused concentration-dependent vasoconstriction. This vasoconstriction was significantly attenuated in STZ-diabetic rats. This attenuated response was further reduced by chronic treatment with L-arginine, although acute L-arginine (10^{-4} M) treatment had no significant effect (Fig. 4, upper panel). In the presence of 10^{-4} M L-NOARG, the ET-1-induced vasoconstriction did not differ between untreated diabetic rats and chronic L-arginine-treated diabetic rats (Fig. 4, lower).

Methoxamine (10^{-7} to 3 \times 10^{-4} M) induced a concentration-dependent vasoconstriction in the mesenteric arterial bed that was weaker in STZ-induced diabetic rats than in the age-matched controls (Fig. 5, upper). Unlike the ET-1-induced vasoconstriction, methoxamine-induced vasoconstriction was not further attenuated by chronic l-arginine treatment in the diabetic rats. Acute l-arginine treatment to diabetic mesentery also had no effect on the methoxamine-induced vasoconstriction. In the presence of 10^{-4} M L-NOARG, the methoxamine-induced vasoconstriction was weaker in STZ-induced diabetic rats than in control rats. Overall, chronic treatment with l-arginine significantly increased the methoxamine-induced vasoconstriction compared with that seen in untreated diabetic rats; this enhancement was due to increased responses in the lower half of the methoxamine concentration-range used (Fig. 5, lower).
The major finding of the current study is that chronic oral administration of L-arginine to STZ-induced diabetic rats resulted in a decrease in plasma ET-1 levels and a greatly attenuated vasoconstrictor response to ET-1. Our data suggest a possible therapeutic role for L-arginine in pathophysiological states associated with altered ET-1 function.

It has been reported that in human aortic endothelial cells prolonged exposure to an elevated concentration of glucose increases endothelial NO synthase (eNOS) gene and protein
expression and NO release (Cosentino et al., 1997). In an in vivo study, the expressions of eNOS and urinary NOx were increased in kidney tissue in STZ-induced diabetic rats (Sugimoto et al., 1998). In our present study, the plasma NOx level was higher in STZ-induced diabetic rats than in control rats, suggesting that the impaired endothelium-dependent vasodilatation seen in STZ-induced diabetic rats is not due to a decreased production of NO. NO activity is reduced by superoxide anions (by chemical interaction and metabolism) and several investigators have noted improved relaxation of arterial vessels after acute incubation with superoxide dismutase (Hattori et al., 1991; Diederich et al., 1994; Rosen et al., 1995; Kamata and Kobayashi, 1996). In vitro experiments have shown that continuous NO production inhibits the synthesis of ET-1 (Boulanger and Lucher, 1990) and that acute inhibition of NO synthesis results in an increase in the ET-1 levels (Richard et al., 1995; Filep, 1997). It is likely, therefore,
that the increase in the plasma ET-1 level seen in diabetic rats is due to an accelerated inactivation of NO. This conclusion is supported by our finding that chronic administration of L-arginine, a precursor of NO, resulted in a significantly higher plasma NOx concentration and a lower plasma ET-1. Although an enhanced inactivation of NO by superoxide anions may occur
in the diabetic state (Kobayashi and Kamata, 1999, 2001), the increase in NO production induced by chronic L-arginine treatment may overcome this enhanced metabolism, thereby leading to a low plasma concentration of ET-1.
In the present study, ET-1 (10⁻⁸ M) caused a transient vasodilatation followed by a marked vasoconstriction in the mesenteric arterial bed preconstricted with methoxamine. We recently reported that in the mesenteric arterial bed, ET-1-induced vasodilatation was mediated by ET₄ receptors localized on the endothelium, while ET-1-induced vasoconstriction was mediated by ET₃ receptors localized on the smooth muscle (Makino and Kamata, 1998a). ET-1-dependent vasodilatation may be mediated by one or more substances including cyclooxygenase product(s) (Lal et al., 1996), NO (Magazine and Srivastava, 1996) and endothelium-derived hyperpolarizing factor(s) (Sakuma et al., 1993). Previous studies suggest that NO functionally antagonizes the effect of ET-1 (Lerman et al., 1995). We recently reported (1) that the marked increase in plasma glucose that occurs in STZ-induced diabetic rats elevates the plasma ET-1; and (2) that the decreased contractile and vasodilator responses of the mesenteric arterial bed to ET-1 in STZ-induced diabetes is due to a desensitization of not only ET₄, but also ET₃ receptors (Makino and Kamata, 1998a,b; Makino and Kamata, 2000; Makino et al., 2001). In the present study, we confirmed that the vasoconstriction induced by ET-1 was significantly attenuated in STZ-diabetic rats. This attenuated response was further reduced by chronic treatment with L-arginine without a change in the sensitivity of the smooth muscle (since there was no difference in the ET-1-induced vasoconstriction in the presence of L-NOARG between untreated diabetic rats and chronic L-arginine-treated diabetic rats). These results suggest that the reduction in the ET-1-induced vasoconstriction seen in L-arginine-treated diabetic rats may result from excess production of NO. Indeed, markedly attenuated ET-1 response in L-arginine-treated diabetic rats was slightly increased in the presence of L-NOARG. Contrast to chronic L-arginine treatment, acute treatment of mesenteric arterial bed with L-arginine did not affect the attenuated ET-1 response in the diabetic rats, suggesting that chronically excess production of NO may be important in ET-1 response. Recent studies have underscored the importance of NAD(P)H-oxidase-derived reactive oxygen species in vascular biology. Many components of the leucocyte-NADPH-oxidase complex — including p22phox, p47phox, p67phox and gp91phox (or a related homologue) — have been identified in endothelial cells or vascular smooth muscle cells (Jones et al., 1996; Bayraktutan et al., 1998; Gorlach et al., 2000; Patterson et al., 1999; Ushio-Fukai et al., 1996). Reactive oxygen species from sources other than NADPH oxidase, such as xanthine oxidase (Adkins and Taylor, 1990) or cytochrome P-450 (Bysani et al., 1990), may also play a role in blood vessels. We have recently reported that the expression of mRNA for p22phox NADH/NADPH oxidase subunit is significantly increased in STZ-induced diabetic rats and this increase can be completely prevented by chronic administration of the endothelin-receptor antagonist J-104132 (Kanie and Kamata, 2002). This suggests that in STZ-induced diabetic rats, ET-1 may be directly involved in impairing endothelium-dependent relaxation via increased superoxide-anion production. Thus, further attenuation of ET-1 response by the chronic administration of L-arginine may be beneficial for diabetic induced vascular disease.

In contrast, in diabetic rats subjected to chronic L-arginine treatment the methoxamine-induced vasoconstriction did not differ from that seen in untreated diabetic rats. In the presence of L-NOARG, chronic treatment with L-arginine significantly increased the methoxamine-induced vasoconstriction compared with that seen in untreated diabetic rats. There are two possible explanations for these results. The first is that NO specifically suppresses the
vasoconstrictor response induced by ET-1, and the second is that in the case of the methoxamine-induced response, a decrease in the vasoconstrictor response to NO is masked by a marked increase in the sensitivity of smooth muscle to methoxamine. Further work on this point will be necessary before we can fully understand our present data.

ET-1 may play roles in various pathological states, including renal dysfunction (Rabelink et al., 1996), atherosclerosis (Hasdai et al., 1997), cerebral vasospasm (Cosentino and Katusic, 1994) and diabetes (Collier et al., 1992; Takahashi et al., 1990, Tsunoda et al., 1991). In general, diabetes mellitus is associated with complications such as renal dysfunction, atherosclerosis and heart failure (Poston and Taylor, 1995). Thus, ET-1 could represent a new marker for diabetes-related vascular disease. In the present study, we have demonstrated that chronic administration of L-arginine markedly lowered the plasma ET-1 level, suggesting that L-arginine treatment could be beneficial in reducing the risk of cardiovascular disease in diabetic mellitus. Indeed, oral administration of L-arginine restores endothelial dysfunction via increased production of NO (Pieper et al., 1996).

In conclusion, chronic L-arginine administration not only reduces the increase in the plasma ET-1 levels otherwise seen in STZ-induced diabetic rats but also decreases the vasoconstriction induced by ET-1. The present data raise the possibility that chronic L-arginine administration may prove to be therapeutically useful for the treatment of vascular complications in diabetes.

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**References**


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