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Short communication

Short-term oral administration of L-arginine reverses defective endothelium-dependent relaxation and cGMP generation in diabetes

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Abstract

In the present study, we evaluated whether acute dietary supplementation with L-arginine in vivo could reverse the defective endothelium-dependent relaxation in diabetic blood vessels assessed ex vivo. At 8 weeks of diabetes, streptozotocin-induced diabetic rats were given 1.25% L-arginine in drinking water 3 days prior to isolation of aortic rings for evaluation ex vivo. Plasma arginine concentration was reduced by diabetes but restored to normal in diabetic rats receiving dietary L-arginine. In norepinephrine-contracted rings, relaxation to acetylcholine but not to nitroglycerin was reduced by diabetes. Dietary treatment with L-arginine restored relaxation to acetylcholine without altering relaxation to nitroglycerin and restored the defect in acetylcholine-stimulated cGMP generation. These data suggest that the substrate for nitric oxide synthesis by the endothelium is likely to be limited in diabetes but can be overcome by dietary supplementation with L-arginine.

Keywords: Endothelium; Nitric oxide (NO); Diabetes mellitus; Arginine

1. Introduction

There is amble evidence that endothelium-dependent relaxation but not endothelium-independent relaxation (to nitrovasodilators) is selectively impaired in blood vessels obtained from chronic diabetic animals (see, for review, Pieper and Gross, 1991; Cohen, 1993; Kamata et al., 1992; Poston and Taylor, 1995). The selective defect in endothelium-dependent relaxation has now been confirmed in Type I (Johnstone et al., 1993; McNally et al., 1994) and Type II (McVeigh et al., 1992) diabetic patients.

Endothelium-dependent relaxation is believed to be mediated, in part, or in whole by release of nitric oxide (NO) or a closely related compound from the endothelial cell (Palmer et al., 1988). NO synthase catalyzes the enzymatic conversion of arginine to citrulline and NO. Thus, it is possible that a defect in arginine supply for NO production by diabetic endothelium could play a role in impaired endothelium-dependent relaxation.

In the absence of any previous study, we evaluated whether short-term oral supplementation with L-arginine in vivo could restore defective endothelium-dependent relaxation in chronic diabetic blood vessels. Furthermore, we investigated whether improvement in endothelial function by increasing dietary arginine was associated with improvement in cGMP generation.

2. Materials and methods

Adult male Sprague-Dawley rats (90 days of age) were anesthetized with an intraperitoneal injection of 60 mg/kg sodium pentobarbital. Diabetes was induced by an intravenous tail-vein injection of streptozotocin (55 mg/kg in 0.1 M citrate buffer, pH 4.5). Diabetic and age-matched control rats were housed for 8 weeks. At 3 days prior to sacrifice, a subset of diabetic rats received 1.25% arginine in drinking water similar to the concentration used in the Dahl/Rapp rat (Chen et al., 1993). On the day of study, rats were anesthetized with 65 mg/kg sodium pentobarbital. Blood samples were taken for analysis of plasma amino acids, blood glucose and glycosylated hemoglobin as described (Pieper et al., 1995).

2.1. Ex vivo analysis of endothelial function

Descending thoracic aortae were carefully isolated and placed in 4°C Krebs bicarbonate buffer. Segments of aor-

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tae were carefully cleaned of fat and loose connective tissue and sectioned into 3 mm long rings. Rings were suspended at an optimal tension of 2.0 g between parallel hooks in 10-ml tissue baths in Krebs-Henseleit medium as previously described (Pieper and Peltier, 1995; Pieper et al., 1995). Changes in isometric tension were recorded on a Gould TA6000 recorder via Radnoti force-displacement transducers. At the end of each experiment, the rings were blotted dry, weighed and the lengths were measured to calculate tension development as normalized for cross-sectional area based upon the formula: cross-sectional area (mm^2) = weight (mg) × [length (mm) × density] with the density of vascular tissue being 1.05 mg/mm^3 (Abebe et al., 1990).

Each ring was exposed to increasing concentrations of norepinephrine. Stock solutions of norepinephrine contained ascorbate to prevent autooxidation (final concentration = 20 nM in the bath). After generating concentration-response curves, each ring was serially washed to baseline tension and equilibrated. For each ring, the pD_2 ($-\log EC_{50}$, the concentration producing 50% maximal response) for norepinephrine was calculated. Rings were then contracted with a submaximal concentration of norepinephrine which elicited approximately 70% of the maximum response. The agonist concentration was usually 1 μM but was varied, if necessary, to achieve equieffective agonist activity.

At the plateau of contraction, relaxation responses to cumulative concentrations of acetylcholine or nitroglycerin to test endothelium-dependent and -independent relaxation, respectively. Only one vasodilator was used for each ring preparation. A few of the vasodilator experiments were conducted in the presence of 100 μM L-nitroarginine to verify the NO dependency of acetylcholine-induced relaxation in these preparations.

2.2. cGMP analysis

Rings were equilibrated in phosphate-buffered saline for 45 min. Thereafter, 100 nM norepinephrine was added followed by 10 μM acetylcholine. The reaction was terminated 1 min later by the addition of trichloroacetic acid followed by extraction in water-saturated ether. cGMP was determined by radioimmunoassay (PerSeptive Diagnostics).

2.3. Statistical analysis

Data are expressed as the mean ± S.E.M. Data were analyzed by analysis of variance followed by Fisher's projected least squares difference test for multiple mean comparisons, unpaired *t*-test for comparisons of two group means, or paired *t*-test for comparisons of two group means. Statistical significance was set at a value of $P < 0.05$.

3. Results

Blood glucose concentrations in diabetic animals were significantly increased compared to control animals (i.e., control: 61 ± 3 mg/dl; diabetic: 396 ± 12 mg/dl; $P < 0.001$). In vivo supplementation with L-arginine for 3 days did not produce a significant change in plasma glucose concentrations at the time of sacrifice (i.e., blood glucose concentration = 386 ± 36 mg/dl). Total glycosylated hemoglobin was elevated in untreated diabetic (i.e., 14 ± 1%) and arginine-treated diabetic (i.e., 13 ± 1%) animals compared to control animals (i.e., 4 ± 1%).

Diabetes produced decreases in the plasma concentration of the basic amino acids, arginine and lysine (Table 1). Concentrations of neutral aromatic amino acids, phenylalanine and tyrosine, were unaltered in diabetic plasma while tryptophan was decreased. The concentrations of other neutral amino acids were either unaltered (e.g., methionine, histidine and threonine) or decreased (e.g., alanine, glycine, cysteine and serine) or elevated (e.g., leucine, isoleucine and valine) by diabetes. The decrease in plasma arginine concentration in diabetic rats was restored by dietary arginine. Some (but not all) of other plasma amino acids were also increased by dietary arginine.

For contractile responses to norepinephrine, there was no significant difference in the maximal tension development (control: 1.82 ± 0.16 g/mm^2; diabetic: 2.01 ± 0.19 g/mm^2) or pD_2 (control: 6.7 ± 0.1; diabetic: 6.7 ± 0.1). Neither the maximum tension development (i.e., 1.78 ±

Table 1
Plasma amino acids

Amino acids	Control ($n = 7$)	Diabetic ($n = 8$)	Diabetic + L-arginine ($n = 7$)
Basic			
Arginine	193 ± 12	115 ± 26 [b]	371 ± 35 [b,d]
Lysine	473 ± 33	268 ± 40 [b]	797 ± 117 [b,d]
Aromatic neutral			
Phenylalanine	76 ± 34	115 ± 21	191 ± 12 [b,d]
Tyrosine	91 ± 6	106 ± 19	159 ± 30 [b]
Tryptophan	96 ± 5	49 ± 12 [b]	n.d.
Branched-chain neutral			
Isoleucine	125 ± 4	467 ± 53 [b]	621 ± 73 [b,c]
Leucine	221 ± 8	843 ± 105 [b]	1155 ± 126 [b,c]
Valine	276 ± 11	1211 ± 133 [b]	1476 ± 238 [b]
Neutral			
Alanine	504 ± 27	366 ± 70 [a]	n.d.
Glycine	322 ± 16	223 ± 19 [a]	192 ± 44 [a]
Serine	211 ± 12	149 ± 19 [a]	n.d.
Threonine	275 ± 16	244 ± 35	n.d.
Cysteine	31 ± 4	40 ± 12	55 ± 15
Methionine	59 ± 2	80 ± 14	146 ± 6 [b,d]
Histidine	72 ± 2	93 ± 18	212 ± 16 [b,d]

Values (in μM) are the mean ± S.E.M.; n.d, not detected. [a] $P < 0.025$, [b] $P < 0.01$ vs. control, [c] $P < 0.05$ and [d] $P < 0.01$ vs. untreated diabetic.

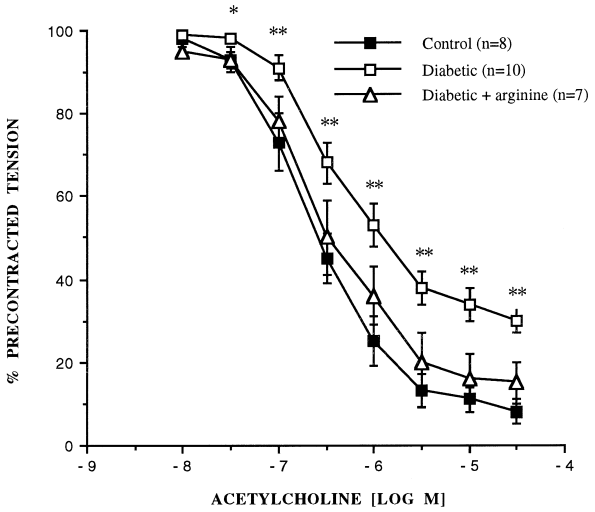

Fig. 1. Impaired relaxation to acetylcholine in diabetic rings and restoration following short-term dietary intake of arginine by diabetic rats in vivo. * $P < 0.05$ and ** $P < 0.01$ vs. control rings.

0.15 g/mm^2) nor pD_2 (i.e., 6.6 ± 0.1) for norepinephrine were altered by dietary L-arginine treatment of diabetic animals.

Acetylcholine-stimulated relaxation was reduced in diabetic rings compared to age-matched control rings (Fig. 1). The maximum relaxation was $92 \pm 3\%$ and $70 \pm 3\%$ in control and diabetic rings, respectively. The pD_2 for acetylcholine was significantly different ($P < 0.05$) between control (6.5 ± 0.1) and diabetic rings (5.9 ± 0.1). In contrast, relaxation to nitroglycerin was unaltered by diabetes (not shown). Supplementation with dietary L-arginine restored the defective relaxation to acetylcholine in diabetic rings (Fig. 1) but did not alter the relaxation to nitroglycerin (not shown). The maximum relaxation (e.g., $85 \pm 5\%$) and pD_2 for acetylcholine (e.g., 6.4 ± 0.1) in rings from arginine-treated rats was significantly different ($P < 0.05$) in rings obtained from untreated diabetic but not control rats. Addition of L-nitroarginine blocked relaxation to acetylcholine by 100 ± 4, 91 ± 2 and $90 \pm 4\%$ in rings from control, untreated diabetic and arginine-treated diabetic rats, respectively.

cGMP production by acetylcholine was reduced in diabetic rings (i.e., 1.8 ± 0.1 pmol/mg protein, $n = 8$) compared to control rings (i.e., $3.6 \pm$ pmol/mg protein, $n = 6$). In rings from arginine-treated rats, cGMP production was restored to control levels (i.e., 4.6 ± 0.6 pmol/mg protein, $n = 5$).

4. Conclusions

While chronic dietary arginine has been shown to partially restore endothelium-dependent relaxation in atherosclerosis (Böger et al., 1995; Cooke et al., 1992), we provide in this study the first known evidence that relatively short-term oral supplementation with arginine can ameliorate defective endothelium-dependent relaxation in diabetic blood vessels. These results are consistent with previous studies in our laboratory and elsewhere showing that acute administration in vitro with L-arginine could also restore endothelial function in aorta of the streptozotocin-induced diabetic rat (Pieper and Peltier, 1995; Pieper et al., 1995) and in coronary arteries of the alloxan-induced diabetic dog (Matsunaga et al., 1996). Taken together, these studies suggest a species-independent defect in an arginine/NO pathway in diabetic endothelium.

Since arginine treatment in vivo did not alter relaxation to nitroglycerin, this suggests that the improvement in endothelium-dependent relaxation to acetylcholine could not be explained by increased sensitivity of diabetic vascular smooth muscle to NO. Furthermore, it is not likely that arginine alters vascular reactivity by supplying substrate for any putative inducible NO synthase in diabetic vascular smooth muscle since we have previously demonstrated in both streptozotocin-induced diabetic (unpublished observations) and genetic BB rats (Pieper et al., 1996) that acute administration of aminoguanidine in vitro fails to alter either contractile tension or relaxation to acetylcholine.

Our studies are consistent with the hypothesis that the supply of arginine for NO synthesis may be compromised in diabetic blood vessels. Indeed, we have confirmed in the present study that plasma arginine concentration is reduced by diabetes in agreement with results previously demonstrated in this laboratory (Pieper and Peltier, 1995; Pieper et al., 1995) and elsewhere in experimental diabetes (Mans et al., 1987) and in human diabetic patients (Grill et al., 1992; Hagenfeldt et al., 1989). This reduction was not due to a generalized reduction in amino acids levels since the concentrations of several neutral amino acids were either unaltered or even increased. Thus, it is possible that decreases in plasma arginine could reduce tissue stores since the plasma arginine concentration in untreated diabetic rats is near the K_m for arginine transport recently reported to be 100 μM in human endothelial cells (Sobrevia et al., 1995). Thus, it is likely that arginine transport would not be saturated under such conditions leading to frank deficiencies in arginine which could be restored by increasing plasma arginine levels.

Interestingly, oral supplementation with arginine also increased lysine (another basic amino acid) as well as several other neutral amino acids. The reason for this increase is unknown but is supported by recent studies showing that arginine administration can influence other amino acid concentrations (Noeh et al., 1996).

We considered the possibility that dietary arginine supplementation might improve endothelium-dependent relaxation by improving the glycemic state of diabetic animals since it is known that arginine administration under certain conditions can elicit insulin release from the pancreas (Schmidt et al., 1992). Furthermore, we have previously shown that improvement in glycemic control following

islet transplantation restores endothelial function in diabetic animals (Pieper et al., 1995). We discount this possibility since total glycosylated hemoglobin and blood glucose concentration were comparable in both untreated diabetic animals and in animals receiving arginine supplementation.

Since endothelium-dependent relaxation to acetylcholine but not endothelium-independent relaxation to nitroglycerin was improved in diabetic aorta in rats receiving dietary arginine, this suggests that replenishment of arginine supply for NO synthase may be the cause and that arginine is likely to be limiting for NO production in diabetes. To further strengthen this conclusion, we performed additional studies showing that defects in acetylcholine-stimulated cGMP production in diabetic aortic rings was normalized in aortic rings from arginine-treated diabetic rats. These observations are consistent with our recent finding that acute treatment ex vivo with L-arginine restored the defective acetylcholine-stimulated cGMP production in diabetic rings. Thus, our studies confirm that oral arginine supplementation does, in fact, augment agonist-stimulated NO production. To our knowledge, this is the first study to demonstrate that a drug or dietary intervention in vivo can improve cGMP production in diabetic blood vessels.

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References

Abebe, W., K.H. Harris and K.M. MacLeod, 1990, Enhanced contractile responses of arteries from diabetic rats to α_1-adrenoceptor stimulation in the absence and presence of extracellular calcium, J. Cardiovasc. Pharmacol. 16, 239.

Böger, R.H., S.M. Bode-Böger, A. Mügge, S. Kienke, R. Brandes, A. Dwenger and J.C. Frölich, 1995, Supplementation of hypercholesterolaemic rabbits with L-arginine reduces the vascular release of superoxide anions and restores NO production, Atherosclerosis 117, 273.

Chen, P.Y., P.L. St. John, K.A. Kirk, D.R. Abrahamson and P.W. Sanders PW, 1993, Hypertensive nephrosclerosis in the Dahl/Rapp rat. Initial sites of injury and effect of dietary L-arginine supplementation, Lab. Invest. 68, 174.

Cohen, R.A., 1993, Dysfunction of vascular endothelium in diabetes mellitus, Circulation 87 (Suppl. 5), V67.

Cooke, J.P., A.H. Singer, P. Tsao, P. Zera, R.A. Rowan and M.E. Billingham, 1992, Antiatherogenic effects of L-arginine in the hypercholesterolemic rabbit, J. Clin. Invest. 90, 1168.

Grill, V., O. Björkman, M. Gutniak and M. Lindqvist, 1992, Brain uptake and release of amino acids in nondiabetic and insulin-dependent diabetic subjects: important role of glutamine release for nitrogen balance, Metabolism 41, 28.

Hagenfeldt, L., G. Dahlquest and B. Persson, 1989, Plasma amino acids in relation to metabolic control in insulin-dependent diabetic children, Acta Pediatr. Scand. 794, 278.

Johnstone, M.T., S.J. Creager, K.M. Scales, J.A. Cusco, B.K. Lee and M.A. Creager, 1993, Impaired endothelium-dependent vasodilation in patients with insulin-dependent diabetes mellitus, Circulation 88, 2510.

Kamata, N., N. Miyata, T. Abiru and Y. Kasuya, 1992, Functional changes in vascular smooth muscle and endothelium of arteries during diabetes mellitus, Life Sci. 50, 1379.

Mans, A.M., R. DeJoseph, D.W. Davis and R.A. Hawkins, 1987, Regional amino acid transport into brain during diabetes: effect of plasma amino acids, Am. J. Physiol. 253 (Endocrinol. Metab. 16), E575.

Matsunaga, T., K. Okumura, H. Ishizaka, R. Tsunoda, S. Tayama, T. Tabuchi and H. Yasue, 1996, Impairment of coronary blood flow regulation by endothelium-derived nitric oxide in dogs with alloxan-induced diabetes, J. Cardiovasc. Pharmacol. 28, 60.

McNally, P.G., P.A.C. Watt, T. Rimmer, A.C. Burdern, J.R. Hearnshaw and H. Thurston, 1994, Impaired contraction and endothelium-dependent relaxation in isolated resistance vessels from patients with endothelium-dependent diabetes mellitus, Clin. Sci. 87, 313.

McVeigh, G.E., G.M. Brennan, G.D. Johnston, B.J. McDermott, L.T. McGrath, W.R. Henry, J.W. Andrews and J.R. Hayes, 1992, Impaired endothelium-dependent and independent vasodilation in patients with type 2 (non-insulin-dependent) diabetes mellitus, Diabetologia 35, 771.

Noeh, F.M., A. Wenzel, N. Harris, L. Milakofsky, J.M. Hofford, S. Pell and W.H. Vogel, 1996, The effects of arginine administration on the levels of arginine, other amino acids and related amino compounds in the plasma, heart, aorta, vena cava, bronchi and pancreas of the rat, Life Sci. 58, PL131.

Palmer, R.M., D.S. Ashton and S. Moncada, 1988, Vascular endothelial cells synthesize nitric oxide from L-arginine, Nature 333, 664.

Pieper, G.M. and G.J. Gross, 1991, Endothelial dysfunction in diabetes, in: Cardiovascular Significance of Endothelium-Derived Vasoactive Factors, ed. G.M. Rubanyi (Futura, Mount Kisco, NY) p. 223.

Pieper, G.M. and B.A. Peltier, 1995, Amelioration by L-arginine of a dysfunctional arginine/nitric oxide pathway in diabetic endothelium, J. Cardiovasc. Pharmacol. 25, 397.

Pieper, G.M., M. Jordan, M.B. Adams and A.M. Roza, 1995, Syngeneic pancreatic islet transplantation reverses endothelial dysfunction in experimental diabetes, Diabetes 44, 1106.

Pieper, G.M., G. Moore-Hilton and A.M. Roza, 1996, Evaluation of the mechanism of endothelial dysfunction in the genetically-diabetic BB rat, Life Sci. 58, PL147.

Poston, L. and P.D. Taylor, 1995, Endothelium-mediated vascular function in insulin-dependent diabetes mellitus, Clin. Sci. 88, 245.

Schmidt, H.H.H.W., T.D. Warner, K. Ischii, H. Sheng and F. Murad, 1992, Insulin secretion from pancreatic β cells caused by L-arginine-derived nitrogen oxides, Science 255, 721.

Sobrevia, L., P. Cesare, D.L. Yudilevich and G.E. Mann, 1995, Diabetes-induced activation of system y^+ and nitric oxide synthase in human endothelial cells: association with membrane hyperpolarization, J. Physiol. 489, 183.