Biochemical Pharmacology, Vol. 46, No. 11, pp. 2097–2099, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

Plasma L-arginine levels in a rabbit model of hypercholesterolaemia

(*Received* 24 *June* 1993; *accepted* 16 *August* 1993)

Abstract—A novel method for the quantitation of L-arginine in plasma samples has been developed, based on the technique of enantiomer labelling coupled to the capillary gas chromatographic separation of amino acids. Using this technique plasma L-arginine levels have been measured in rabbits fed a 1% cholesterol-containing diet and compared with rabbits fed a standard diet. Blood cholesterol levels were significantly elevated in the rabbits fed cholesterol but no significant difference was found in plasma L-arginine levels between the cholesterol-fed and control animals. No D-arginine was found in any of the plasma samples. It is concluded that the impairment in endothelium-dependent relaxation previously described in this animal model of hypercholesterolaemia is not due to deficiency of plasma L-arginine.

Hypercholesterolaemia in animal models and humans is accompanied by endothelial dysfunction, which is manifest as an attenuation of endothelium-dependent vasorelaxation [1]. The exact mechanism underlying this dysfunction is unclear [2].

Endothelium-derived relaxing factor, now known to be nitric oxide (NO) or a NO-containing compound that liberates NO [3, 4], is derived from the terminal guanidino nitrogen of L-arginine [5]. Recent reports have shown that it is possible to correct the endothelial dysfunction associated with hypercholesterolaemia by administration of L-arginine [6–10]. This would suggest that the impaired endothelium-dependent relaxation may be related to L-arginine deficiency, though the findings of a recent *in vitro* study would seem to contradict this [11].

Analysis of the L-arginine content of plasma from hypercholesterolaemic patients has also produced conflicting results. One study has shown reduced plasma L-arginine levels in these patients as compared to controls [12] whereas other studies using similar groups of patients failed to find any difference in their L-arginine levels [13, 14].

We have attempted to resolve this problem in the present study by comparing plasma L-arginine levels in rabbits fed a normal or a high cholesterol-containing diet. In doing so we have developed a novel method for the quantitation of L-arginine based on the technique of enantiomer labelling [15] coupled to the capillary gas chromatographic separation of amino acids as their pentafluoropropionyl derivatives. The technique of enantiomer labelling overcomes the inherent problems of poor precision and accuracy associated with previously published gas chromatographic techniques for the quantitation of amino acids in biological fluids [16].

Materials and Methods

Chemicals. L-Arginine, D-arginine as their hydrochloride salts and acetylchloride were obtained from Merck Chemicals (Poole, U.K.). Dichloromethane, HPLC grade was obtained from Rathburn Chemicals Ltd (Walkerburn, U.K.) and pentafluoropropionic anhydride from the Aldrich Chemical Co. (Gillingham, U.K.). The chromatographic column was purchased from Alltech UK (Carnforth, U.K.).

Animals and treatments. Male New Zealand white rabbits (2–2.5 kg, N = 22) were randomly assigned to two groups. In one group 12 received a standard diet and in the other 10 received a diet uniformly enriched with 1% cholesterol. Both groups of rabbits were allowed *ad lib.* access to the appropriate food for 8 weeks.

At the end of the 8 week period the rabbits were killed by cervical dislocation. Blood samples were taken via cardiac puncture into plain glass tubes and then centrifuged at 500 *g* for 10 min to isolate the plasma.

Assay of plasma cholesterol. The plasma cholesterol levels were measured using a standard Technicon RA-1000 system. In this system cholesterol esterase is used to hydrolyse cholesterol esters in the plasma samples to free cholesterol. The free cholesterol is then oxidized to produce hydrogen peroxide that in turn is used to form a quinoneimine dye. The concentration of the dye, measured colorimetrically at 500 nm, is directly proportional to the cholesterol content of the plasma sample.

Assay of plasma L-arginine. For calibration purposes 0.5 mL aliquots of fresh rabbit plasma were taken and 0.05 mL aliquots of standard L-arginine at concentrations of 0.948, 0.474 and 0.237 mmol/L prepared in 0.2 M HCl. In experimental samples the addition of standard L-arginine was replaced by an equal volume of 0.2 M HCl. D-Arginine (0.05 mL) at a concentration of 0.474 mmol/L prepared in 0.2 M HCl was added to each calibration and experimental sample. Plasma proteins were precipitated by the addition of 2 mL of ice-cold acetone, vortexed briefly for 5 sec and centrifuged at 1400 *g* for 5 min at 4°. The supernatant was removed and one drop of concentrated HCl and 2 mL of chloroform added. The sample was rotary mixed for 5 min and then centrifuged as before. Of the aqueous upper layer, 0.4 mL was transferred to a clean glass tube and evaporated to dryness at 100° under a stream of air. The extracted amino acids were then esterified by adding 0.5 mL of a freshly prepared isopropanol:acetylchloride (4:1) mixture, capped and placed in a heating block at 100° for 30 min. The esterification agent was then evaporated to dryness at 100° under a stream of air and the tube cooled. Amino acids were derivatized by the addition of 0.4 mL of dichloromethane and 0.1 mL of pentafluoroproprionic anhydride, capped and heated at 100° for 30 min. The derivatization reagent was evaporated to dryness under air and the sample reconstituted by vortexing for 10 sec with 0.2 mL of dichloromethane. Aliquots of 0.1 mL were then transferred into disposable glass inserts placed in autosampler vials and crimped ready for autoinjection.

Chromatographic conditions. Separation and quantitation of L-arginine were performed using a Hewlett packard 5890 gas chromatograph incorporating a 7673A autosampler, a 3390A integrator and a standard flame ionization detector. The chromatographic column used was a 25 m × 0.32 mm i.d. Heliflex fused silica capillary column coated with a Chirasil-valine stationary phase at a thickness of 0.2 μm. The carrier gas was oxygen-free nitrogen set at an inlet pressure of 5 psi and the gases for the flame ionization detector, air and hydrogen, were set at inlet pressures of 40 and 20 psi, respectively. Injection volumes of 1 μL were

Table 1. Plasma L-arginine and cholesterol concentrations (mmol/L) of rabbits fed on either a normal or a 1% cholesterol-enriched diet.

	Normal diet		1% Cholesterol diet	
	L-arginine	Cholesterol	L-arginine	Cholesterol
	0.18	2.00	0.23	56.00
	0.22	0.91	0.24	67.20
	0.23	0.96	0.29	39.10
	0.38	1.08	0.23	54.10
	0.30	1.17	0.20	62.30
	0.25	1.30	0.43	44.70
	0.34	0.92	0.38	65.30
	0.24	1.33	0.55	37.80
	0.21	2.01	0.28	71.70
	0.21	0.79	0.36	63.70
	0.38	1.39		
	0.26	1.99		
Mean	0.27	1.32	0.32	56.19
± SEM	0.02	0.13	0.04	3.81
N	12	12	10	10

made by the injector set in the splitless mode. The injection and detector temperatures were set at 250°.

The temperature programme developed to achieve effective resolution of the D- and L-arginine isomers was: initial temperature 50° held for 4 min. This temperature was then increased to 150° at a rate of 5°/min and held for 10 min. The final operating temperature of 200° was reached at a rate of 4°/min and held for an additional 10 min, whereafter the temperature returned to 50° for the next sample injection. The overall run time was 56.5 min.

Quantitation of L-arginine. By virtue of the fact that L-arginine is an endogenous component of plasma, quantitation is based upon a combination of both standard addition and internal standardization. Firstly, the ratios, calculated by dividing the integrated peak height of the calibration material containing added quantities of L-arginine by the integrated peak height of added D-arginine, are plotted against the added concentrations of L-arginine and subjected to linear regression analysis. The negative intercept of the X axis being the endogenous L-arginine concentration of the calibration material. This line is then forced through the "zero" intercept and the slope used to convert the integrated peak height ratios of experimental samples into endogenous L-arginine concentrations.

Statistics. Data are expressed as means ± SEM. Comparisons were made using the unpaired t-test and differences considered significant when $P < 0.05$.

Results and Discussion

Using enantiomer-labelling, i.e. D-arginine as the internal standard, intra-assay coefficients of variation (CVs) of 3.2% and 4.6% and inter-assay CVs of 4.8% and 5.4% were obtained for samples containing 0.05 mmol/L of L-arginine.

In normal plasma samples the retention time for L-arginine was 40.129 min. In plasma samples spiked with D-arginine, the retention time for D-arginine was 39.769 min.

Blood cholesterol levels were significantly increased in the rabbits fed on the high cholesterol diet (56.19 ± 3.8 mmol/L; N = 10) compared to those fed on the normal diet (1.32 ± 0.13 mmol/L; N = 12; $P < 0.01$). However, there was no significant difference between the plasma L-arginine levels of either group of rabbits (0.32 ± 0.04 mmol/L; N = 10 and 0.27 ± 0.02 mmol/L; N = 12, respectively) (see Table 1). No D-arginine was detected in any of the samples measured. Likewise, no D-arginine has been detected in human plasma samples using this method (unpublished observations).

It has previously been reported that aortae from the cholesterol-fed rabbits show impaired endothelium-dependent responses to acetylcholine and ATP, whereas responses to nitroglycerin [17] or sodium nitroprusside [18] are either unaltered or enhanced. The overall effect of impaired agonist-induced NO release on vascular tone in atherosclerotic vessels is not clear since other neurohormonal (e.g. catecholamines) and hormonal (e.g. prostaglandins, endothelins) influences may play a role in vascular tone regulation *in vivo*.

The results of the present study show that the plasma L-arginine levels in the hypercholesterolaemic rabbits are not significantly different from those of normal rabbits. This supports the previously reported studies in humans [13, 14] and rabbits [19], and suggests that the impaired endothelium-dependent responses in hypercholesterolaemia are not due to L-arginine deficiency.

In the previous studies where human plasma L-arginine levels were assessed [12–14] it was not clear whether L-arginine was distinguished from D-arginine, and indeed whether the latter was measured at all. The present study demonstrates a method for the specific measurement of L-arginine by virtue of being able to resolve the enantiomers. Using this method no D-arginine was found in the plasma samples of either group of rabbits studied.

The question still remains as to how administration of L-arginine can restore the endothelial dysfunction associated with hypercholesterolaemia. If this dysfunction is not due to a lowering of plasma L-arginine levels it may result from depletion of L-arginine in the endothelial cells themselves. Endothelial cells take up L-arginine by the saturable cationic transport system y^+, the latter being inhibited by the NO synthase inhibitor n^G-monomethyl-L-arginine and other cationic amino acids [20]. Furthermore, bradykinin and ATP have been shown to stimulate simultaneous uptake of L-arginine by this transport system and the release of NO [21]. It is therefore possible that hypercholesterolaemia may impair the y^+ system leading to a substrate deficiency and reduced NO production. The administration of excess L-arginine may then override this impairment thus restoring normal endothelial function.

It has been shown in other cell types that L-arginine transport appears to depend upon the membrane potential [22, 23]. This has not however been established for

endothelial cells. If this was also the case for endothelium, a hypercholesterolaemia-induced alteration in the membrane potential could result in a disrupted L-arginine transport system. Further work is necessary to substantiate this possibility.

In conclusion, the novel assay method developed in this study shows that the major proportion of arginine in the plasma from both the control and the hypercholesterolaemic rabbits is of the L-form. More importantly it showed the levels of L-arginine in the two groups of rabbits to be identical. The exact mechanism by which the administration of L-arginine can reverse the hypercholesterolaemia-induced endothelial dysfunction remains unclear and requires further investigation.

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