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A NOVEL CITRULLINE-FORMING ENZYME IMPLICATED IN THE FORMATION OF NITRIC OXIDE BY VASCULAR ENDOTHELIAL CELLS

Richard M.J. Palmer and Salvador Moncada

Wellcome Research Laboratories
Langley Court, Beckenham, Kent BR3 3BS, U.K.

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SUMMARY: An enzyme in homogenates of porcine vascular endothelial cells forms L-citrulline from L-arginine. This enzyme is soluble and NADPH-dependent. In addition, the enzyme is inhibited by N^G-monomethyl-L-arginine, suggesting that it is involved in the formation of nitric oxide by vascular endothelial cells.

Vascular endothelial cells synthesise nitric oxide (NO) from the terminal guanidino nitrogen atom(s) of L-arginine (1) in amounts sufficient to account for the biological activity on vascular strips (2) and platelets (3,4) attributed to endothelium-derived relaxing factor. The formation of NO by vascular endothelial cells and the endothelium-dependent vascular relaxation are inhibited by N^G-monomethyl-L-arginine (L-NMMA; 5,6). Cytotoxic macrophages form NO_2^- and NO_3^-, the oxidation products of NO, from L-arginine with the concomitant formation of L-citrulline (7,8). The generation by macrophages of both NO_2^- and NO_3^- and of L-citrulline is also inhibited by L-NMMA (9).

In view of this we have investigated the formation of L-citrulline from L-arginine by homogenates of vascular endothelial cells and have partially characterised the enzyme responsible for this reaction.

MATERIALS AND METHODS

Enzyme preparation: Fresh porcine thoracic aortae were obtained from a local abattoir and dissected free of adherent fat and connective tissue. The lumen was washed with sterile phosphate buffered saline (pH 7.2) and the aorta opened longitudinally. The endothelium was removed and homogenised with the flat of a sterile scalpel blade and the homogenate transferred to ice cold 0.1 M Tris buffer, pH 7.2 (2 aortae/ml buffer). The homogenate was then sonicated 3 times for 10 s and kept on ice. In some experiments the homogenate was centrifuged at 105,000 g for 1 h and the supernatant used as a source of soluble enzyme.

Porcine aortic endothelial cells were isolated and cultured on microcarriers as described previously (10). The cells were washed five

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times in Krebs buffer before being removed from the microcarriers by vigorous shaking in ice-cold 0.1 M Tris buffer pH 7.2. The supernatant was removed, sonicated as above and used as the source of enzyme.

Citrulline formation: The reactions were initiated by addition of endothelial cell homogenate (0.2 ml) to a mixture containing 5 μCi [^{3}H]L-arginine (sp. act. 57 Ci/mmol; Amersham International) and the appropriate compounds described below in a total volume of 0.5 ml 0.1 M Tris buffer, pH 7.2. After incubation at 37°C for 10 min, the reaction was stopped by addition of 0.1 ml 2.5 M perchloric acid and the tubes kept at 4°C until assayed. An aliquot (0.1 ml) was fractionated by ion exchange high pressure liquid chromatography on BTC 2710 with discontinuous gradient elution on an amino acid analyser (Biotronik LC 6001). The radioactivity present in 1 min fractions of the effluent was determined by liquid scintillation counting and the retention times of L-arginine, L-ornithine and L-citrulline were determined with appropriate standards.

RESULTS AND DISCUSSION

The formation of [^{3}H]citrulline was not observed when [^{3}H]arginine was incubated with NADPH (1.5 mM) alone and only minimal amounts were detectable when the endothelial homogenate was incubated with [^{3}H]arginine (Fig. 1a, b). However, [^{3}H]citrulline formation from [^{3}H]arginine was enhanced when NADPH, but not NADP, NADH or NAD (all at 1.5 mM), was incubated with the homogenate (Fig. 1c). The formation of [^{3}H]ornithine or other [^{3}H]amino acids was not detectable under these conditions, indicating that the homogenates did not contain detectable arginase activity, and that [^{3}H]citrulline was only formed from [^{3}H]arginine by the NADPH-dependent enzyme. The enzyme was located in the 105,000 g supernatant of the homogenate (n=3), with no detectable activity in the washed pellet, showing that it is either a soluble enzyme or that it is only loosely associated with membranes. This enzyme was also found in homogenates of porcine aortic endothelial cells in culture which had been shown, using a bioassay technique (2), to release NO (n=3).

The formation of [^{3}H]citrulline was inhibited by 71.5 ± 8.9 % (mean ± s.e.m., n=3) by L-NMMA (3 μM; Fig. 1d), but not by its D-enantiomer (30 μM). These results strongly indicate that the enzyme is involved in the formation of NO from L-arginine in vascular endothelial cells, for this process is also inhibited by L-NMMA (5,6). The fact that L-NMMA appears to be a more potent inhibitor of [^{3}H]citrulline formation than of endothelial NO formation (5) or endothelium-dependent relaxation (6) reflects the competitive nature of its action since only low concentrations of L-arginine were present in the homogenates of endothelial cells (< 12.5 μM by amino acid analysis). In preliminary experiments we failed to detect by chemiluminescence (2) the formation of NO or its oxidation product NO_2^-. Whether this is due to the generation of an intermediate which is not further

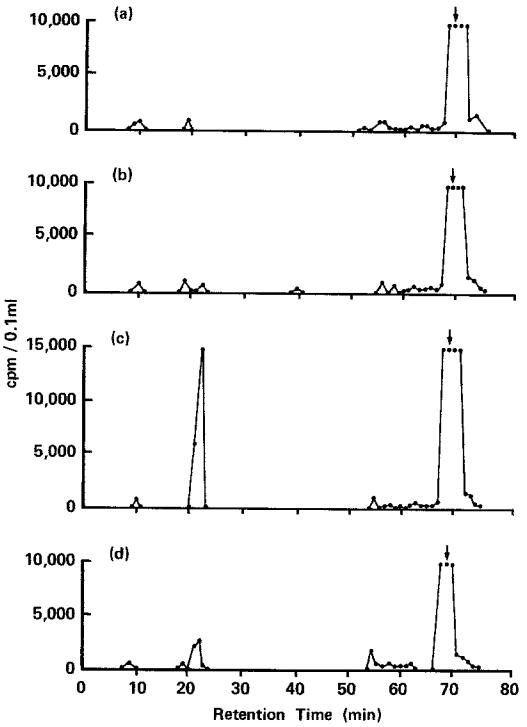

Fig. 1

Elution profile from the HPLC of incubates of [^{3}H]arginine with (a) NADPH, (b) endothelial homogenate alone, (c) endothelial homogenate and NADPH, (d) endothelial cell homogenate, NADPH and L-NMMA (3 μM) from the same representative experiment in which the protein concentration was 2.5 mg/ml. The retention times of L-citrulline, L-ornithine and L-arginine were 22, 58 and 69 min respectively. indicates the [^{3}H]arginine peak.

converted to NO under these conditions or is due to haemoglobin in the homogenate, which binds NO and would mask its presence, is not clear.

L-arginine (100 μM), but not D-arginine (100 μM), inhibited the formation of [^{3}H]citrulline from [^{3}H]arginine by 89.0 ± 1.3 % (n=3). In contrast, neither L-citrulline (100 μM) nor L-canavanine (100 μM), which inhibits the synthesis of citrulline by macrophages (8), affected [^{3}H]citrulline formation. These results confirm our previous suggestion (1) that [^{3}H]citrulline is not a substrate for the NO-forming enzyme in endothelial cells and that this enzyme differs in this respect from that in the macrophage.

High concentrations of L-canavanine have been reported to inhibit endothelium-dependent relaxation in rat aortic rings (11) and the synthesis of NO from L-arginine in bovine aortic endothelial cells (12). The discrepancy between these results and our own may indicate species differences, for we have observed that L-canavanine does not affect the

formation of NO in the perfused rabbit aorta (6). The fact that L-canavanine is inactive against the soluble enzyme suggests that this, rather than a problem of uptake, is the explanation for the differences observed.

The citrulline-forming enzyme in macrophages has been suggested to be a deiminase which generates ammonia that is then oxidised to NO_2^- and NO_3^- (7). Endothelial cells and cytotoxic macrophages do form ammonia (13,14), however, it is not clear whether a deiminase or another mechanism is involved in this process. Furthermore, deiminases have not been identified in mammalian cells and it would be energetically wasteful to reduce the imino nitrogen atom of L-arginine prior to oxidation to NO. In view of this, further evidence is required to elucidate the precise nature of the enzyme(s) involved in the formation of NO by vascular endothelial cells.

In the present experiments we did not observe peptidyl-citrulline in the HPLC profile, indicating that [^{3}H]arginine is converted directly to [^{3}H]citrulline by the soluble fraction of endothelial cell homogenates. This observation suggests that L-arginine is not incorporated into a peptide prior to conversion to NO. This excludes the involvement of peptidyl-arginine deiminase in [^{3}H]citrulline formation (15).

The formation of [^{3}H]citrulline by endothelial cell homogenates was not affected by superoxide dismutase (15 $U.ml^{-1}$; n=3) suggesting that the endothelial cell enzyme differs from that responsible for citrulline formation in murine haemopoietic cells (16). The haemopoietic cell enzyme is dependent on oxygen radicals, is inactive in arginine-free medium and has been suggested to be a superoxide-dependent peptidyl-arginine deiminase (17).

[^{3}H]citrulline formation was inhibited by methylene blue (59.4 ± 5.7 % n=3) at 25 μM, but not by ascorbate (5 mM). This, together with the requirement for NADPH, strongly implicates a redox mechanism in this process. The formation of [^{3}H]citrulline was marginally inhibited by high concentrations of cyanide (18.9 ± 10.4 %, n=3) at 1 mM, but not by bubbling the enzyme with CO for 5 min, suggesting that it is not a haem enzyme but that it does require a cation.

The requirement for some divalent cations by the enzyme was examined by incubating the homogenate with Ca^{2+} (2.5 mM), Mg^{2+} (0.8 mM), Fe^{2+} and Fe^{3+} (both at 0.1 mM) in the presence of NADPH. None of these cations enhanced the formation of [^{3}H]citrulline further (n=3). EDTA was also used to determine whether sufficient divalent cations were already present in the homogenate for maximum activity of the enzyme. [^{3}H]citrulline formation was inhibited by 68.9 ± 7.3 % (n=3) by 0.2 mM EDTA. This finding suggests that there is a divalent cation requirement for enzyme activity and further work is required to identify which one. Since the release of NO by

endothelial cells is Ca^{2+}-dependent (18), it is possible that this cation is involved. Interestingly, the enzyme in the macrophage shows some requirement for Mg^{2+} (19).

In summary, we have presented evidence for the existence of a novel NADPH-dependent enzyme in vascular endothelial cells, which may be involved in the formation of NO from L-arginine. In many respects this enzyme is similar to that in macrophages. The presence and biological relevance of this enzyme in tissues other than vascular endothelial cells and macrophages should be investigated.

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REFERENCES

1. Palmer, R.M.J., Ashton, D.S. and Moncada, S. (1988) Nature, 333,664-666.
2. Palmer, R.M.J., Ferrige, A.G. and Moncada, S. (1987) Nature, 327, 524-526.
3. Radomski, M.W., Palmer, R.M.J. and Moncada, S. (1987) Br. J. Pharmac., 92, 639-646.
4. Radomski, M.W., Palmer, R.M.J. and Moncada, S. (1987) Biochem. Biophys. Res. Commun., 148, 1482-1489.
5. Palmer, R.M.J., Rees, D.D., Ashton, D.S. and Moncada, S. (1988) Biochem. Biophys. Res. Commun., 153, 1251-1256.
6. Rees, D.D., Palmer, R.M.J., Hodson, H.F. and Moncada, S. Br. J. Pharmac., in press.
7. Hibbs, J.B., Taintor, R.R. and Vavrin, Z. (1987) Science, 235, 473-476.
8. Iyengar, R., Stuehr, D.J. and Marletta, M.A. (1987) Proc. Natl. Acad. Sci. USA, 84, 6369-6373.
9. Hibbs, J.B., Vavrin, Z. and Taintor, R.R. (1987) J. Immunol., 138, 550-565.
10. Gryglewski, R.J., Moncada, S. and Palmer, R.M.J. (1986) Br. J. Pharmac., 87, 685-694.
11. Schmidt, H.H.H.W., Klein, M.M., Niroomand, F. and Bohme, E. (1988) Europ. J. Pharmac., 148, 293-295.
12. Schmidt, H.H.H.W., Nau, H., Wittfoht, W., Gerlach, J., Prescher, K-E., Klein, M.M., Niroomand, F. and Bohme, E. (1988) Europ. J. Pharmac., 154, 213-216.
13. Truskey, G.A. and Davies, P.F. (1985). Cell Biol. Int. Rep., 9, 323-330.
14. Currie, G.A. (1978) Nature, 273, 758-759.
15. Thomas, G. and Ramwell, P.W. (1988) Biochem. Biophys. Res. Commun., 154, 332-338.
16. Schneider, E., Kamoun, P.P., Migliore-Samour, D. and Dy, M. (1987). Biochem. Biophys. Res. Commun., 144, 829-835.
17. Kamoun, P.P., Schneider, E. and Dy, M. (1988). FEBS Lett., 226, 285-286.
18. Singer, H.A. and Peach, M.J. (1982) Hypertension, 4(Suppl. II), 19-25.
19. Iyengar, R. and Marletta, M.A. (1988) Biochemistry, 27, 3096.