Biochemical Pharmacology, Vol. 38, No. 11, pp. 1709–1715, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00

COMMENTARY

BIOSYNTHESIS OF NITRIC OXIDE FROM L-ARGININE

A PATHWAY FOR THE REGULATION OF CELL FUNCTION AND COMMUNICATION

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We have recently demonstrated that nitric oxide (NO), which accounts for the biological activities of endothelium-derived relaxing factor (EDRF), is synthetized by the vascular endothelium from the amino acid L-arginine. This and other evidence, emerging from unrelated fields of research, is revealing the existence of a biochemical pathway for the formation of NO, the main function of which is the stimulation of the soluble guanylate cyclase.

The information available is sufficiently strong to warrant the conclusion that this pathway is a widespread system for the regulation of cell function and communication. This system underlies a variety of biological actions which include endothelium-dependent relaxation, cytotoxicity of phagocytic cells, and cell-to-cell communication in the central nervous system. The biological significance of this pathway will be discussed in this commentary.

The vascular endothelium

Endothelium-derived relaxing factor [1] is a labile humoral substance which relaxes vascular smooth muscle and inhibits platelet aggregation and adhesion via stimulation of the soluble guanylate cyclase. The chemical nature of EDRF has now been identified as NO. The pharmacological properties of EDRF and NO are identical (for review see Ref. 2), including their ability to elevate cGMP levels in vascular tissue [3]. Furthermore, NO is released from vascular endothelial cells in culture [4, 5] and from vascular preparations [3, 6–9] in amounts sufficient to explain the biological actions of EDRF.

Porcine vascular endothelial cells in culture synthetize NO from the terminal guanido nitrogen atom(s) of L-arginine [10]. This reaction, which has also been reported by others [11], is specific, since a number of analogues of L-arginine, including its D-enantiomer, are not substrates. In addition, one analogue, L-N^G-monomethyl arginine (L-NMMA), inhibits this synthesis in a dose-dependent and enantiomerically-specific manner [12]. The co-product of this reaction is L-citrulline, for its synthesis from L-arginine by vascular endothelial cell homogenates is also inhibited by L-NMMA [13].

Several lines of evidence indicate that L-arginine is the physiological precursor for the formation of NO in vascular tissue and that NO is involved in the control of vascular tone. In rabbit aortic rings, L-NMMA induces a small but significant endothelium-dependent contraction and inhibits the relaxation and the release of NO induced by acetylcholine (ACh). L-Arginine, which on its own only induces a small endothelium-dependent relaxation, antagonizes all the actions of L-NMMA [14]. Similar results have been obtained by Sakuma *et al.* [15] in guinea pig pulmonary artery rings and by ourselves [16] in the coronary circulation of the rabbit heart *in vitro*.

More significantly, in the anesthetized rabbit L-NMMA induces a dose-dependent long-lasting increase in mean arterial blood pressure and inhibits the hypotensive action of ACh without affecting that of the endothelium-independent vasodilator, glyceryltrinitrate. These effects of L-NMMA are reversible by L-arginine (Fig. 1; [17]).

These results clearly indicate that there is, in the vasculature, a continuous utilization of L-arginine for the generation of NO which plays a role in the maintenance of blood pressure. This release of NO can be enhanced further by endothelium-dependent vasodilators. Furthermore, the marked rise in blood pressure obtained after inhibition of NO synthesis confirms the proposal that NO is the endogenous nitrovasodilator (for review see Ref. 18) and suggests that a reduction in the synthesis of NO may contribute to the genesis of hypertension.

The release of NO by the vascular endothelium may also play other important regulatory roles in the cardiovascular system. To date there is only one report that endogenous NO inhibits platelet function *in vivo* [19] although it is a potent inhibitor of platelet function *in vitro* (for review see Ref. 18). Endogenous NO has also been implicated in the modulation of mesangial cell contractility [20] and control of the release of renin [21]. Whether NO also modulates vascular smooth muscle cell replication or regulates enzymes involved in the metabolism of cholesterol requires investigation.

The macrophage

The association between nitrosamines and cancer and the possibility that nitrosamines are formed from NO_3^- by gut microorganisms led to the study of NO_3^- metabolism in humans. The excess NO_3^-

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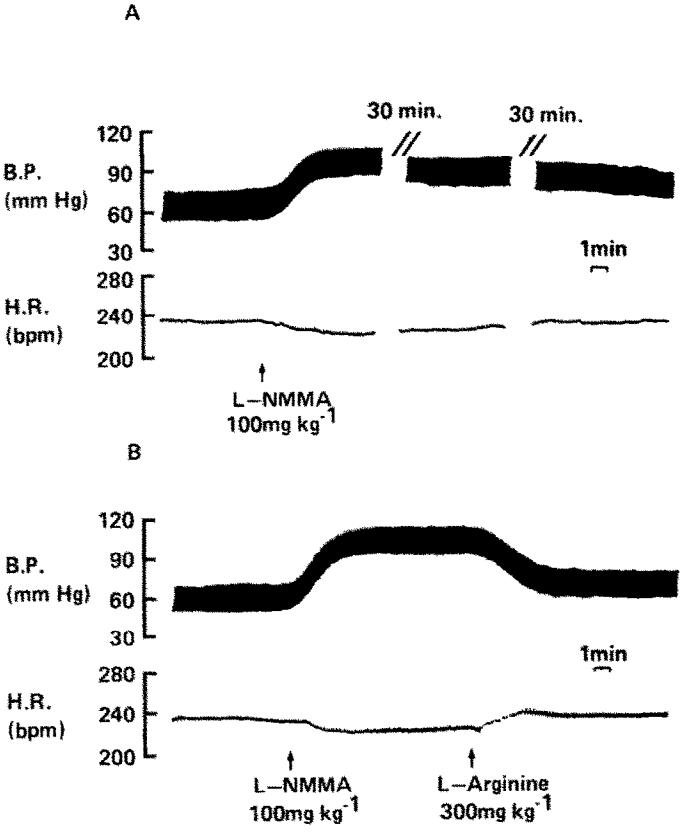

Fig. 1. (A) Effect of L-NMMA (100 mg kg^{-1}, administered as a 15-sec infusion i.v.) on blood pressure (B.P.) and heart rate (H.R.) in the anesthetized rabbit. (B) Reversal of the effect of L-NMMA (100 mg kg^{-1}, 15-sec infusion, i.v.) on blood pressure and heart rate by L-arginine (300 mg kg^{-1}, 15-sec infusion, i.v.). Data were taken, with permission from Ref. 17.

excreted by mammals on low NO_3^- diets, which was first believed to be a product of intestinal microbial metabolism, was later demonstrated, in germ-free animals, to be mammalian in origin [22]. A marked increase in urinary NO_3^- excretion was also observed in human subjects with diarrhea [23]. Subsequently, it was demonstrated that treatment of rats with *Escherichia coli* lipopolysaccharide (LPS), turpentine or carrageenin led to an increase in the urinary output of NO_3^-, suggesting that this was a consequence of activation of the reticulo-endothelial system [24].

Later work showed that blood levels and urinary excretion of NO_3^- increased after exposure to LPS in LPS-sensitive mice [25] and that activated mouse peritoneal macrophages show increased NO_2^- and NO_3^- production *in vitro* [25]. Moreover, L-arginine is essential for the formation of NO_2^- and NO_3^- which occurs after activation of the macrophage cell line RAW 264.7 with LPS and γ-interferon [26]. The NO_2^- and NO_3^- formed are derived from the terminal guanido nitrogen atom(s) of L-arginine. This reaction, which is independent of the respiratory burst, results in the formation of L-citrulline as a co-product [26].

An L-arginine-dependent pathway, distinct from arginase [27], has been shown to be responsible for the cytotoxic activities of macrophages [28]. These cytotoxic activities, which include inhibition of mitochondrial respiration, aconitase activity and DNA synthesis, are thought to be mediated by inhibition of iron-containing enzymes in target cells. These activities, as well as the generation of NO_2^- and NO_3^-, are inhibited by L-NMMA [28]. Tumor necrosis factor increases macrophage NO_3^- production in response to γ-interferon, and this effect is also inhibited by L-NMMA [29]. Interestingly, the formation of NO_2^- and NO_3^- from L-arginine seems to occur in viable macrophages, whereas lysis or extensive damage to the macrophages is required for arginase to be expressed [30].

Although the generation of NO* by these cells has not yet been demonstrated, it is likely that NO_3^- and NO_2^- synthesis proceeds via the formation of NO. The functions of this NO generation, other than as a cytotoxicity mechanism, are not known; however, it is possible that NO may regulate cGMP levels in cells with which the macrophage comes in contact. Furthermore, it is also possible that the generation of NO may alter the intracellular environment of the macrophage by the induction of specific changes in

* Since this manuscript was submitted the generation of NO by macrophages has been shown by three groups [31–33].

metabolism which facilitate its microbiostatic activity [34]. Whether this is subsequent to the elevation of cGMP or is an independent mechanism requires investigation.

The release of an NO-like material has also been detected from peritoneal neutrophils [35], which are themselves inhibited from aggregating by NO [36]. Since NO exerts powerful biological effects in different tissues and is generated by vascular tissue and by inflammatory cells, it is likely that the formation of NO from L-arginine is involved in the genesis and development of the signs and symptoms of inflammation.

The central nervous system

Acetylcholine and other muscarinic agents elevate cGMP levels in the brain. However, they fail to stimulate soluble guanylate cyclase in cell-free systems, suggesting that these agents release a factor that in turn activates the enzyme.

In 1977, the soluble fraction of brain synaptosomes was shown to contain a low molecular weight activating factor of the soluble guanylate cyclase. This factor was different from all previously known activators of this enzyme and its action was prevented by hemoglobin [37]. Since the effects of the activator and of nitroso compounds were not additive and both were inhibited by *N*-methylhydroxylamine and hydroxylamine, it was speculated that this activator may be related to the nitrosamines or may be acting at the same allosteric site [38]. Subsequently, the endogenous activator was identified as L-arginine [39] and, independently, NO was shown to stimulate the soluble guanylate cyclase in homogenates of mouse cerebral cortex [40].

In view of this, we investigated whether the brain contains the enzyme which transforms L-arginine into NO. These studies showed that in rat brain synaptosomal homogenates L-arginine was converted to L-citrulline with a concomitant synthesis of NO which increases cGMP (Knowles, Palacios, Palmer and Honcada, submitted for publication). Both of these processes were inhibited by L-NMMA, indicating that the enzyme system involved is similar to that in the endothelial cell and in the macrophage [12, 13, 28, 29].

While this work was in progress, Garthwaite *et al.* [41] reported that *N*-methyl-D-aspartate (NMDA), an excitatory amino acid known to elevate cGMP levels in the brain, induces the release of an EDRF-like material from rat cerebellar cells. This factor is unstable, and its stability is enhanced by superoxide dismutase. Furthermore, it relaxes vascular smooth muscle, and this effect is inhibited by hemoglobin. The release of this factor, which is Ca^{2+}-dependent, accounts for the elevation in cGMP levels that follows NMDA receptor activation.

Although the soluble guanylate cyclase is present in the central nervous system, it is not uniformly distributed throughout the brain and its role is yet to be understood. Cholinergic and adrenergic transmission, excitatory amino acids, some other amines, depolarizing agents, and peptides have been shown to elevate cGMP (for review see Ref. 42). It is possible that in many of these cases receptor-mediated activation of the formation of NO from L-arginine leads to stimulation of the soluble guanylate cyclase. The biological consequences of stimulating the soluble guanylate cyclase in different parts of the brain are yet to be elucidated. As far as excitatory amino acids are concerned, it is possible that not only their normal biological functions but also their convulsant and their "excitotoxic" actions are mediated through this mechanism, since the last two could be a consequence of Ca^{2+} overload [43, 44] which may result from overstimulation of the cGMP system.

Characteristics of the L-arginine:NO-forming enzyme system

Present evidence indicates that in all the tissues investigated thus far L-arginine is enzymically converted into NO with the concomitant formation of L-citrulline (Table 1).

The tissues from which most information is currently available are the vascular endothelium, the macrophage and the central nervous system. In other tissues, the evidence, although less complete, also points to the existence of the L-arginine:NO pathway. The formation of NO has been demonstrated recently in neutrophils [35], and we have found that this formation is inhibited by L-NMMA, a process that can be reversed by L-arginine [47]. Furthermore, EMT-6 adenocarcinoma cells, activated with supernatant fraction from macrophages in culture, generate L-citrulline, NO_2^- and NO_3^- from L-arginine [46]. Whether or not the recently described formation of L-citrulline from L-arginine in murine hematopoietic cells also occurs via this pathway has not been established [49]. It has recently been shown that Kupffer cells decrease hepatocyte protein synthesis via an arginine-dependent mechanism [50]. The formation of NO by mouse liver has already been demonstrated [51], suggesting that the L-arginine:NO-forming enzyme may also occur in this tissue.

The similarities between the systems from different sources are remarkable when analyzed in terms of substrate specificity, products and cofactor requirements (Table 1). The enzymes in the vascular endothelium, the macrophage and the central nervous system are soluble, NADPH-dependent, require a divalent cation and are inhibited by L-NMMA [13, 28, Knowles, Palacios, Palmer and Moncada, submitted for publication]. At present it is not possible to tell whether they are the same enzyme or a system of isoenzymes. The latter alternative is more likely since the enzymes from the different cells show small variations in their substrate specificity and in their susceptibility to inhibition by L-arginine analogues.

The exact mechanism whereby the terminal guanido nitrogen atom(s) of L-arginine is liberated and subsequently oxidized to NO is not known. In the macrophage, an L-arginine deiminase that liberates ammonia, which is then oxidized to NO_2^- and NO_3^-, has been postulated [52]. So far the term deiminase is being used for a reaction in which the imino nitrogen is removed, without a rigorous identification of the enzyme [30, 52]. However, deiminases have only been clearly identified in bacteria [53], and other mechanisms such as N-oxidation may be involved [31]. Furthermore, the NADPH-depen-

Table 1. Substrate/inhibitor profile for the enzymes involved in the L-arginine:NO pathway

	EC [10, 14]	EH [13]	Mo [26, 28, 31–33, 45]	CNS [41,*]	EMT-6 [46]	PMN [47]
Substrate						
L-Arginine	+	+	+	+	+	+
D-Arginine	0	0	0	0	0	0
L-Homoarginine	0	+	+	+	ND	+
α-Amino γ-guanidobutyric acid	0	0	ND	0	ND	ND
L-Citrulline	0	0	0	0	0	0
L-Arg-Asp	0	+	+	+	ND	+
L-Arg-methyl ester	0	ND	+	+	ND	0
Benzoyl L-arginine ethyl ester†	0	0	ND	0	ND	ND
Inhibitor						
L-NMMA	+	+	+	+	ND	+
D-NMMA	0	0	ND	0	ND	0
L-Canavanine	0	0	+	0	ND	+
Formamidineacetate	0	0	ND	0	ND	ND
Products						
L-Citrulline	ND	+	+	+	+	ND
NO	+	ND	+	+	ND	+
NO_2^-/NO_3^-	ND	ND	+	+/ND	+	ND
Cofactor						
NADPH	ND	+	+	+	ND	ND

Data obtained from references in brackets or from our unpublished observations.

Abbreviations: EC, endothelial cell; EH, endothelial homogenate; Mo, macrophage; CNS, central nervous system; EMT-6, murine adenocarcinoma cells; and PMN, polymorphonuclear leukocytes. Key: (+) active; (0) inactive; ND, not done.

* Knowles, Palacios, Palmer and Moncada, submitted for publication.

† Since benzoyl L-arginine ethyl ester, which relaxes rat aortic rings [48], is not a substrate for the formation of NO from L-arginine, it is unlikely that peptidyl arginine deiminase is involved in this pathway.

dence of the enzymes suggests a redox mechanism. Additional work will be required to clarify this process.

The release of EDRF is known to be dependent on extracellular calcium [54]. The way in which this dependence relates to the mechanism of activation of the enzyme(s) involved in the generation of NO from L-arginine requires elucidation. If this process involves two steps which are receptor activated, it will also be interesting to study the relationship between this and other calcium-dependent pathways.

The precise circumstances and the way in which the L-arginine:NO pathway is activated also remain to be established. A basal and a stimulated release of EDRF/NO have been demonstrated in vascular endothelial cells *in vitro* [55, 56] and in the coronary circulation of the isolated heart [9]. The basal release exhibited by these preparations could simply be the consequence of the *in vitro* situation. However, our experiments in the whole animal [17] indicate the presence of a basal release of NO *in vivo*. Whether this is due to the action of endogenous endothelium-dependent vasodilators or to hemodynamic factors, including the pumping of the heart, needs to be investigated. The possibility that other cells also exhibit a basal and a stimulated release of NO should also be studied.

It is also necessary to investigate the source of L-arginine for the formation of NO, the precise steps involved in this formation, and the fate of NO once it has exerted its biological function. In addition, the importance of this pathway in relation to the utilization of L-arginine in the urea cycle or for protein synthesis remains to be studied.

Other roles for the L-arginine:NO pathway?

The pathways of nitrogen metabolism, including the urea cycle and amino acid metabolism, have been studied extensively for many years. It is therefore surprising that the formation of NO from L-arginine in mammalian tissues and the potential biological significance of this process have not been revealed before.

L-Arginine can either be obtained from the diet or can be synthetized from L-glutamate in reactions involving the urea cycle. Because of this, dietary L-arginine is not indispensable for the survival of most adult mammals; however, it is required for maximum growth in young animals and for normal repair following injury (for review see Ref. 57). When investigating the existence and role of NO formation in different organs, it will be necessary to take into account that L-arginine, besides being the precursor of this pathway, is also a constituent of proteins, participates in amino acid metabolism, and is required for the biosynthesis of polyamines.

There are, however, a number of other biological actions of L-arginine which may be mediated by the L-arginine:NO pathway. Administration of L-arginine induces the release of growth hormone [58], prolactin [59], insulin [60] and glucagon [61] in a number of species both *in vitro* and *in vivo*. L-Arginine also stimulates the release of pancreatic somatostatin [62], pancreatic polypeptide [63],

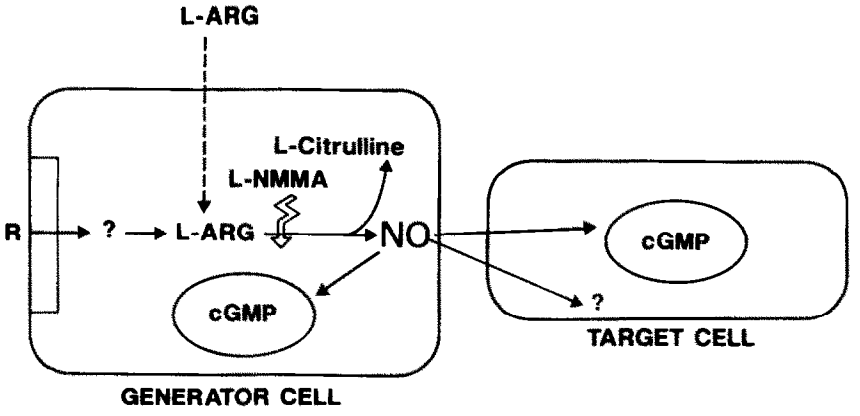

Fig. 2. A schematic diagram of the L-arginine:NO pathway. A question mark (?) indicates areas requiring clarification.

adrenal catecholamines [64] and vasopressin [65]. There is circumstantial evidence to suggest that at least one of these actions of L-arginine may be mediated via the formation of NO, since rats starved for 48 hr show significantly reduced insulin release in response to glucose and also have lowered endogenous levels of cGMP. Furthermore, analogues of cGMP potentiate the insulin response to glucose in pancreatic islets from starved rats, but not in those from fed rats [66]. Interestingly, insulinopenia and a failure to release insulin in response to glucose have been observed in L-arginine-deficient rats [67], suggesting that endogenous L-arginine may be involved in the release of insulin induced by glucose.

L-Arginine increases skin allograft rejection [68] and tumor regression [69] and decreases tumor recurrence in animals [70]. These actions could be a result of the enhancement of NO generation by macrophages or may be due to the thymotropic effect of L-arginine, the mechanism of which is still unknown (for review see Ref. 57).

Finally, supplementation of protein diets with L-arginine is known to decrease cholesterol levels and atherogenesis in animals and humans [71, 72]. Whether this is due to stimulation of NO-dependent pathways or to other actions of L-arginine remains to be investigated.

General conclusions

The existing evidence allows us to postulate that the L-arginine:NO pathway is a widespread transduction mechanism for activation of the soluble guanylate cyclase, leading to a variety of functions in different cells (see Fig. 2). It is also possible that NO performs other functions which are unrelated to the activation of this enzyme. Nitric oxide is a potent nitrosating agent and this could explain some of its biological actions. However, since NO is thought to activate soluble guanylate cyclase through an interaction with the iron atom in the heme of the enzyme and this is also the way in which NO interacts with hemoglobin and aconitase, it is tempting to speculate that all the actions of NO are mediated through an interaction with transition metals.

Although all the work described in this review relates to mammalian cells, endothelium-dependent relaxation has been demonstrated in reptiles [73] and the soluble guanylate cyclase is present in squid giant axons [74], suggesting that the L-arginine:NO pathway may be of an early evolutionary origin.

The precise distribution of the L-arginine:NO pathway in the body remains to be studied and for this a systematic investigation of the biological actions of L-arginine and the correlation between this and the distribution of the soluble guanylate cyclase in tissues will be useful. Since L-NMMA is a specific inhibitor of this pathway *in vitro* and *in vivo*, there is also scope for investigating the biological consequences of removing NO generation.

What is already evident is that the occurrence of the L-arginine:NO pathway indicates a general regulatory function which, if altered, could contribute to the genesis of a wide variety of diseases. Furthermore, it is likely that selective pharmacological manipulation of this pathway may represent a novel approach to the prevention or treatment of some pathophysiological entities.

Acknowledgements—We wish to thank Mrs G. Henderson for her assistance in the preparation of this manuscript.

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