# Nitric Oxide: Physiology, Pathophysiology, and Pharmacology

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## I. Introduction

THE demonstration in 1987 of the formation of NO* by an enzyme in vascular endothelial cells opened up what can now be considered a new area of biological research (for review, see Moncada et al., 1989). NO, which accounts for the biological properties of EDRF, is the endogenous stimulator of the soluble guanylate cy-

*Abbreviations: NO, nitric oxide; EDRF, endothelium-derived relaxing factor; ACh, acetylcholine; Hb, hemoglobin; GMP, guanosine monophosphate; SOD, superoxide dismutase; L-NMMA, N^G-monomethyl-L-arginine; NADPH, nicotinamide adenine dinucleotide phosphate (reduced form); GTN, glyceryl trinitrate; L-NA, N^G-nitro-L-arginine; L-NIO, N-iminoethyl-L-ornithine; L-NAME, N^G-nitro-L-arginine methyl

ester; SNP, sodium nitroprusside; ADP, adenosine diphosphate; NMDA, N-methyl-D-aspartate; NANC, nonadrenergic, noncholinergic; LPS, lipopolysaccharide; IFN-γ, interferon-γ; TNF, tumour necrosis factor; FMLP, formyl-methionyl-leucyl-phenylalanine.

clase. In addition, NO is an effector molecule released by murine macrophages and other cells after immunological activation.

NO is synthesized from the amino acid L-arginine by an enzyme, the NO synthase. In the last year it has become apparent that there are at least two types of this enzyme. One is constitutive, cytosolic, $Ca^{2+}$/calmodulin dependent, and releases NO for short periods in response to receptor or physical stimulation. The NO released by this enzyme acts as a transduction mechanism underlying several physiological responses. The other enzyme is induced after activation of macrophages, endothelial cells, and a number of other cells by cytokines and, once expressed, synthesizes NO for long periods. Furthermore, this enzyme is cytosolic, $Ca^{2+}$ independent, it requires tetrahydrobiopterin as well as other cofactors, and its induction is inhibited by glucocorticoids. So far, the only clearly established role for this NO is as a cytotoxic molecule for invading microorganisms and tumour cells. It is likely, however, that the release of NO via this enzyme has other biological consequences including pathological vasodilation and tissue damage.

This review will consequently be divided into two major areas, one relating to the role of NO as a transduction mechanism and the other to its role as an effector molecule whose release is induced during immunological reactions.

## II. Nitric Oxide as a Transduction Mechanism for the Soluble Guanylate Cyclase

### A. Vasculature

*1. Early observations of endothelium-dependent relaxation and endothelium-derived relaxing factor from 1980 to 1987.* In 1980, Furchgott and Zawadzki demonstrated that the vascular relaxation induced by ACh was dependent on the presence of the endothelium and provided evidence that this effect was mediated by a labile humoral factor, later known as EDRF. Endothelium-dependent relaxation, which was subsequently demonstrated in many vascular preparations, including some veins, arteries, and microvessels, occurs in response to a variety of substances, such as ACh, adenine nucleotides, thrombin, substance P, the calcium ionophore A23187, and bradykinin. Other stimuli, such as hypoxia, increase in flow, and electrical stimulation, also cause endothelium-dependent relaxation of vascular tissue in vitro. Some agents, however, such as the nitrovasodilators, atrial natriuretic factor, bovine retractor penis inhibitory factor, $\beta$-adrenergic agonists, and prostacyclin, induce vascular relaxation by endothelium-independent mechanisms (for reviews, see Furchgott, 1984; Griffith et al., 1984; Busse et al., 1985; Moncada et al., 1986b).

The humoral nature of EDRF was first demonstrated using a variety of pharmacological preparations in which the biologically active substance was transferred from a donor to a detector bioassay. One such system consisted of a "sandwich" arrangement of two rabbit aortic strips in which the EDRF donor (a strip with intact endothelium) was placed, intimal surface to intimal surface, next to the detector (a strip without endothelium; Furchgott, 1984). Another approach involved perfusion of the lumen of an intact rabbit isolated aorta, the effluent of which was used to superfuse endothelium-denuded vascular rings (Griffith et al., 1984; Rubanyi et al., 1985). Stimulation of the donor aorta with ACh caused relaxation of the detector tissues. Finally, vascular endothelial cells, cultured on microcarriers and packed in the barrel of a syringe or a modified chromatography column, were perfused, and the effluent was used to superfuse a ring of canine coronary artery or a series of rabbit aortic strips denuded of endothelium (Cocks et al., 1985; Gryglewski et al., 1986a).

It was established, using techniques such as these, that EDRF was a very short-lived substance with a half-life of only seconds in oxygenated physiological salt solutions (Griffith et al., 1984; Cocks et al., 1985). Release of EDRF was observed under basal conditions as well as after stimulation with ACh (Griffith et al., 1984; Rubanyi et al., 1985; Martin et al., 1985). The effects of EDRF were shown to be inhibited by Hb, methylene blue (Martin et al., 1985), and other agents such as dithiothreitol and hydroquinone (Griffith et al., 1984) and to be mediated by stimulation of the soluble guanylate cyclase with the consequent elevation of intracellular cyclic GMP levels (Rapoport and Murad, 1983).

Bioassay studies in which the source of EDRF, either fresh vascular tissue with endothelium (Rubanyi et al., 1985) or vascular endothelial cells in culture (Cocks et al., 1985; Gryglewski et al., 1986a), was separated from the detector (endothelium-denuded vascular rings or strips) allowed the study of the effects of physical or chemical manipulation on the generation, stability, or actions of EDRF. It was found using such techniques that superoxide anions ($O_2^-$) contribute to the instability of EDRF, because the effects of EDRF were prolonged by the addition of SOD (Gryglewski et al., 1986b; Rubanyi and Vanhoutte, 1986) and inhibited by $Fe^{2+}$ (Gryglewski et al., 1986b) and hyperoxia (Rubanyi and Vanhoutte, 1986). Furthermore, a number of compounds described as inhibitors of EDRF were shown to act by generating $O_2^-$ in solution as a result of their redox properties. Indeed, SOD attenuated their inhibitory effects on the action of EDRF (Moncada et al., 1986a). These observations led to the prediction and subsequent confirmation that another substance capable of removing $O_2^-$, cytochrome $c$, would attenuate the action of these redox compounds on EDRF and that an $O_2^-$-generating compound, pyrogallol, would also act as an inhibitor (Moncada et al., 1986a). This latter compound has now been used by a number of authors to investigate the biological properties of EDRF (Ignarro et al., 1987; Matsunaga and Furchgott, 1989; Shultz and Raij, 1989).

Generation of $O_2^-$, however, does not account for the action of all inhibitors of EDRF, because Hb does not act by this mechanism (Martin et al., 1986; Hutchinson et al., 1987).

EDRF was also shown to inhibit platelet aggregation (Azuma et al., 1986; Furlong et al., 1987; Radomski et al., 1987a), to cause disaggregation of aggregated platelets, and to synergize with prostacyclin in both of these actions (Radomski et al., 1987b). In addition, EDRF inhibits platelet adhesion to endothelial monolayers, extracellular matrix, and collagen fibrils (Radomski et al., 1987c,d).

2. Identification of endothelium-derived relaxing factor as nitric oxide. Early suggestions that EDRF might be a product of the arachidonic acid lipoxygenase (Singer and Peach, 1983; Forstermann and Neufang, 1984) or of the cytochrome P-450 enzyme system (Pinto et al., 1985; Macdonald et al., 1986) or was a compound with a carbonyl group near its active site (Griffith et al., 1984) did not lead to the identification of its chemical structure. Based on the similarities in the pharmacological behaviour of EDRF and NO generated from acidified $NO_2^-$, Furchgott suggested in 1986 that EDRF may be NO (see Furchgott, 1988). At the same time, Ignarro et al. also speculated that it may be NO or a closely related species (see Ignarro et al., 1988).

The first evidence for the formation of NO by mammalian cells came from experiments in which EDRF released from vascular endothelial cells was detected by the chemical means used to identify NO. NO may be measured directly as the chemiluminescent product of its reaction with ozone (Downes et al., 1976). It was shown using this method that the concentrations of bradykinin that induced the release of EDRF from porcine aortic endothelial cells in culture also caused a concentration-dependent release of NO. Moreover, the amounts of NO released by the cells were sufficient to account for the relaxation of vascular strips (Palmer et al., 1987; fig. 1). Furthermore, the levels of NO released by these cells also accounted for the inhibition of platelet aggregation and adhesion induced by EDRF (Radomski et al., 1987b,c). A key element in all of these studies was the correlation between the amounts of NO measured by bioassay and those detected by chemiluminescence.

A detailed comparison of the biological actions of EDRF and NO on vascular strips (Palmer et al., 1987; Hutchinson et al., 1987) and on platelets (Radomski et al., 1987a) also showed that the two compounds were indistinguishable (Moncada et al., 1988b). Both EDRF and NO caused a relaxation of the vascular strips that declined at the same rate during passage down the bioassay cascade (Palmer et al., 1987; fig. 2). Furthermore, the rate of decay during transit in polypropylene tubes was slower but similar for both compounds, indicating that they have identical chemical stability even under these artificial conditions. Both EDRF and NO also inhibited
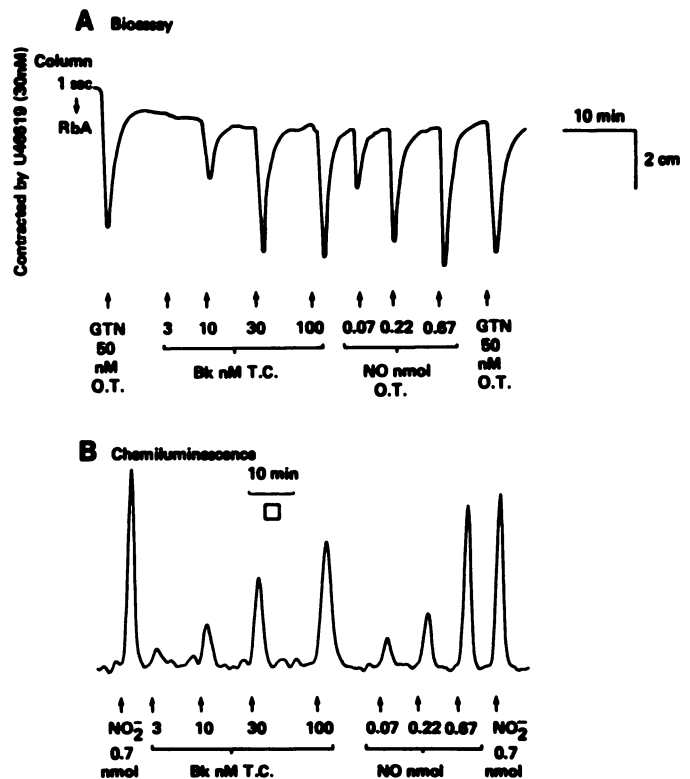


FIG. 1. Correlation between NO measured by bioassay and by chemiluminescence. A, Bioassay: relaxation of rabbit aorta by EDRF and NO. A column packed with endothelial cells cultured on microcarriers was perfused with Krebs' buffer (5 ml/min). The effluent was used to superfuse three spiral strips of rabbit aorta (RbA), denuded of endothelium, in a cascade (top tissue only shown in this figure). The tissues were contracted submaximally by a continuous infusion of 9,11-dideoxy-9$\alpha$, 11$\alpha$-methano epoxy-prostaglandin $F_{2\alpha}$ (U46619; 30 nM) and were separated from the cells by delays of 1, 4, and 7 s, respectively. The response of the detector tissues was calibrated by administration of a standard dose of GTN (50 nM) over the tissues (O.T.). The bioassay tissues were relaxed in a concentration-dependent manner by EDRF released from the cells by 1-min infusions through the column (T.C.) of bradykinin (Bk; 3 to 100 nM) and by NO (0.07 to 0.67 nmol, O.T.) dissolved in He-deoxygenated water and administered as 1-min infusions. B, Chemiluminescence: release of NO by bradykinin (Bk) from a replicate column of the cells used in the bioassay. The amounts of NO (administered as a 1-min infusion into the column effluent) which relaxed the bioassay tissues were also detectable by chemiluminescence. Effluent from the column, or Krebs' buffer into which authentic NO was injected, was passed continuously (5 ml/min) into a reaction vessel containing 75 ml 1.0% sodium iodide in glacial acetic acid under reflux. NO was removed from the refluxing mixture under reduced pressure in a stream of $N_2$, mixed with ozone, and the chemiluminescent product was measured with a photomultiplier. The amounts of NO detected were quantified with reference to a $NO_2^-$ standard curve. □, area equivalent to 0.22 nmol NO. Reprinted with permission from Nature 327: 524–526, 1987 (Macmillan Magazines Ltd.).

platelet aggregation (Radomski et al., 1987a), induced the disaggregation of aggregated platelets (Radomski et al., 1987b), and inhibited platelet adhesion (Radomski et al., 1987c,d). Moreover, their biological half-lives as inhibitors of platelet aggregation were similar (Radomski et al., 1987a).

The actions of EDRF and NO on vascular strips and on platelets were similarly potentiated by SOD and cy-
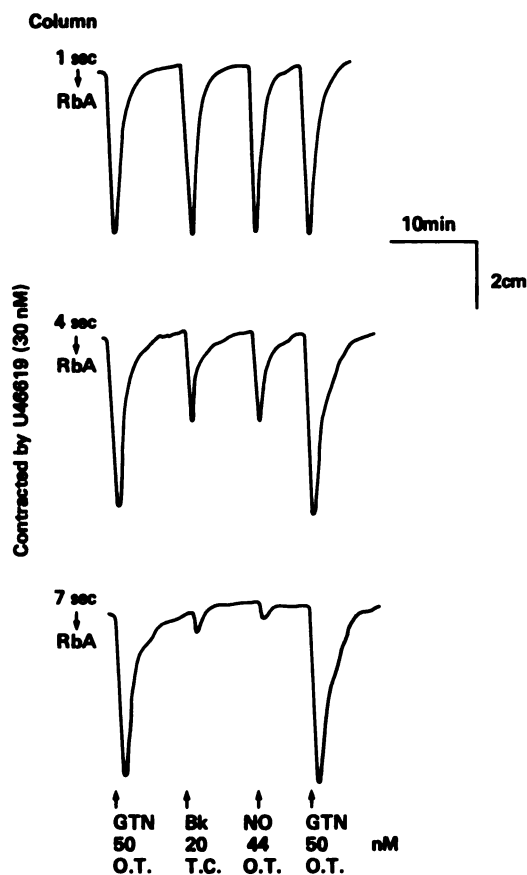
FIG. 2. Comparison of the stability of EDRF and NO during passage down a bioassay cascade. EDRF (released by bradykinin, 20 nM) and NO (44 nM) decay at similar rates. For details of experimental procedure, see legend to fig. 1A. Reprinted with permission from Nature 327: 524–526, 1987 (Macmillan Magazines Ltd.).

tochrome $c$ and inhibited by $Fe^{2+}$ and some redox compounds (Palmer et al., 1987; Hutchinson et al., 1987; Radomski et al., 1987a). Furthermore, the potency of redox compounds as inhibitors of EDRF-induced and NO-induced vascular relaxation was attenuated by SOD to a similar extent. In addition, the inhibitory action of Hb on EDRF can be explained by the fact that this substance binds avidly to NO (Hermann, 1865; Gibson and Roughton, 1957; Martin et al., 1986). Finally, both EDRF and NO act on vascular smooth muscle (Kukovetz et al., 1979; Rapoport and Murad, 1983) and platelets (Mellion et al., 1981) through the stimulation of soluble guanylate cyclase and elevation of cyclic GMP.

NO release from vascular endothelial cells from other species and from a number of vascular preparations, in amounts sufficient to account for the biological actions of EDRF, has also been demonstrated. It was also shown, using a chemical assay based on the diazotization of sulfanilic acid by NO and subsequent coupling with N-(1-naphthyl)-ethylene diamine, that NO or a labile nitroso species was released from perfused bovine pulmonary artery. Furthermore, perfusion of segments of pulmonary artery or pulmonary vein with A23187 caused relaxant responses and elevation of vascular cyclic GMP

levels in the bioassay tissues that could be matched by NO (Ignarro et al., 1987). Similar results were obtained in perfused rabbit aortae stimulated with ACh, A23187, and substance P (Khan and Furchgott, 1987; Chen et al., 1989). Later, the use of a spectrophotometric assay, based on the reaction between NO and Hb, also demonstrated the release of NO from vascular endothelial cells in culture (Kelm et al., 1988). Furthermore, the release of NO from isolated perfused rabbit (Amezcua et al., 1988) or guinea pig (Kelm and Schrader, 1988) hearts has been shown to account for the vasodilator actions of ACh and bradykinin in these preparations.

All of this evidence strongly supported the proposal that EDRF is NO. Moreover, NO fulfilled the criteria for identification as a biological mediator, as originally defined by Dale (1933).

*3. Controversy about the chemical identity of endothelium-derived relaxing factor.* Although the evidence for EDRF being NO is compelling, several lines of research have questioned this conclusion. These include observations about variations in the half-life of EDRF, differential binding of EDRF and NO to anion exchange columns, differential activity of EDRF and NO on smooth muscle preparations, stabilization of EDRF, and poor correlations between biological activity and the amounts of NO detectable by chemical methods.

Wide variations in the half-life of EDRF (from 3 to 50 s; Griffith et al., 1984; Forstermann et al., 1984; Rubanyi et al., 1985; Cocks et al., 1985; Gryglewski et al., 1986b) can probably be explained in terms of the relative contributions of $O_2$ and $O_2^-$ to the inactivation of NO under different conditions. Oxygen is known to react rapidly with NO to form $NO_2$ which in solution forms nitrite ($NO_2^-$) and nitrate ($NO_3^-$), both of which are almost inactive on platelets and vascular strips (Radomski et al., 1987b; Palmer et al., 1987). NO has also been shown to react with $O_2^-$ to form $NO_3^-$ (Blough and Zafiriou, 1985). Thus, different half-lives are likely to be reported as the experimental conditions vary from laboratory to laboratory, making these studies of limited value. To date, no systematic study has been conducted of the chemistry of NO in physiological solutions at 37°C.

EDRF has been reported to bind to anion exchange columns (Cocks et al., 1985; Long et al., 1987), whereas NO does not (Long et al., 1987). However, other workers have shown that amounts of NO that induce comparable relaxation of the bioassay tissues also bind to these columns, although to a lesser extent (Khan and Furchgott, 1987; R. M. J. Palmer and S. Moncada, unpublished results). NO is not anionic, but it is known to nitrosate amines and, therefore, it is likely that the interaction of EDRF and NO with these columns is a chemical reaction.

The response of the bioassay tissues to bolus administration of an agonist differs quantitatively from that to an infusion. The release of NO after stimulation is likely to occur at variable rates for periods in excess of 1 min.

Because of this, the comparison between NO released from cells or tissues and bolus injections of NO may be misleading.

Some authors have reported that EDRF only relaxes vascular smooth muscle, whereas NO relaxes vascular, tracheal, and taenia coli smooth muscle (Shikano and Berkowitz, 1987; Dusting et al., 1988a). These results are difficult to analyse because neither group presented evidence showing that equieffective amounts of EDRF and NO were compared. The guinea pig tracheal strip is approximately 30 times less sensitive to infusions of NO than the rabbit aortic strip, suggesting that the amount of EDRF required for its relaxation is in excess of that released by porcine cells in culture (Dusting et al., 1988b; R. M. J. Palmer and S. Moncada, unpublished results). Furthermore, several laboratories have reported that, under appropriate experimental conditions, EDRF from cultured endothelial cells relaxes the same nonvascular smooth muscle preparations as does NO (Gillespie and Sheng, 1988; Angus and Cocks, 1989; Buga et al., 1989; Furchgott et al., 1990).

EDRF has been reported to be stabilized by acidification (Murray et al., 1986), a condition that would not be expected to stabilize NO. However, this can be explained in terms of the transformation of NO into $NO_2^-$, which can generate NO when acidified. In support of this, it has been shown that $NO_2^-$ has the same chromatographic mobility as that reported (Murray et al., 1986) for the "stable form" of EDRF (R. M. J. Palmer and S. Moncada, unpublished results).

EDRF released from cultured bovine aortic endothelial cells by bradykinin has been found to be much more stable than NO when the effluent from which it was detected was immediately cooled to 0°C (Angus and Cocks, 1989). Furthermore, when the effluent was lyophilized and then reconstituted, it still had a major part of the initial relaxing activity, suggesting that EDRF may be a stabilized precursor of NO. An alterative explanation is that bradykinin stimulated the release from these cultured cells of both NO and a substance (or substances) that reacted with NO to form a much more stable product that can release NO on contact with tissue at 37°C. Such a product may be similar to the inhibitory factor from the bovine retractor penis (Gillespie and Sheng, 1988; Martin et al., 1988). The acid-activatable precursor of this factor is now thought to be $NO_2^-$ (Furchgott, 1988; Martin et al., 1988), although the NO formed on acidification is stabilized even after neutralization by some substance (or substances) also present in the extract.

Recently, EDRF has been suggested to be an unstable nitroso compound, such as S-nitroso-cysteine, based on comparisons of its potency with that of NO on vascular strips and on the dissociation between measurements of NO release made by bioassay and chemiluminescence (Myers et al., 1990). However, the $EC_{50}$ for NO on vascular strips in this study was significantly greater than that reported by other workers. Furthermore, contamination of NO with $NO_2^-$, which can occur in the preparation or use of these solutions (Furchgott, 1990), could account for discrepancies in the measurements of NO by bioassay and by chemiluminescence, which measures both NO and $NO_2^-$. Because these authors report identical stability of NO, EDRF, and S-nitroso-cysteine, it is more likely that differences in potency or in the amounts of NO measured are simply methodological.

Evidence from electron paramagnetic resonance spectroscopy has also recently questioned the identification of NO as EDRF (Rubanyi et al., 1990; Vedernikov et al., 1990). These measurements are based on the reaction between NO and Hb to form paramagnetic nitrosyl-Hb and show that NO, but not EDRF, forms such a species under apparently equivalent conditions determined by bioassay. However, the sensitivity of this method is limited and $NO_2^-$ can also form nitrosyl-Hb under some conditions (Doyle et al., 1988). Because this method is based on a chemical reaction, it is essential to compare NO and EDRF under the same conditions.

The finding that EDRF and NO have identical chemical stability and quantitatively and qualitatively identical biological actions indicates that, if EDRF is released from the endothelial cells as an unstable precursor, it must break down completely within 1 s. Because cell membranes are readily permeable to NO, it is difficult to envisage a role for such a precursor, which may not easily penetrate cell membranes. However, if the existence of such an intermediate is proven, it will not detract from the fact that the biological effects of EDRF are mediated ultimately by NO. Distinction between these options is unlikely to be achieved by bioassay experiments because the responses of tissues to NO, or unstable NO generators, administered under different conditions, cannot be clearly distinguished. It is likely, therefore, that this question will only be resolved when the biochemical mechanism of the synthesis of NO is clarified.

The existence of mechanisms other than NO which play a role in endothelium-dependent relaxation cannot be excluded at present. For example, stimulation of the endothelium of some arteries by ACh results in a hyperpolarization of the adjacent smooth muscle cells which may contribute to their relaxation (Komori and Suzuki, 1987; Feletou and Vanhoutte, 1988; Brunet and Beny, 1989). This hyperpolarization has been attributed to the release of a factor, termed endothelium-derived hyperpolarizing factor (Feletou and Vanhoutte, 1988; Chen et al., 1988; Taylor and Weston, 1988). Although it has been reported that endothelium-derived hyperpolarizing factor differs from NO in that it is not influenced by Hb or methylene blue (Chen et al., 1988; Taylor and Weston, 1988; Chen and Suzuki, 1989), NO has recently been shown to cause hyperpolarization of some arteries (Tare et al., 1990). Further work is required to clarify the

functional significance of hyperpolarization of both endothelial and smooth muscle cells.

The existence of other endothelium-dependent vasodilator mediators would not be surprising, because mechanisms subserving a biological function are usually multiple. It is important to stress, however, that endothelium-dependent relaxations that are susceptible to inhibition by Hb and methylene blue, indicating the involvement of NO and cyclic GMP, have been demonstrated in many vascular preparations, whereas evidence for other mechanisms is scant.

*4. Synthesis of nitric oxide.* Early work ruled out the likelihood of compounds such as $NO_2^-$, $NO_3^-$, $NH_3$, and hydroxylamine being the source of NO (R. M. J. Palmer and S. Moncada, unpublished results). In 1988, however, the amino acid L-arginine was shown to be the precursor for the synthesis of NO by vascular endothelial cells. Endothelial cells, cultured in the absence of L-arginine for 24 h prior to the experiments, showed a decrease in the release of EDRF induced by bradykinin and A23187 which could be restored by L- but not D-arginine (Palmer et al., 1988a). Furthermore, this enhancement only occurred in the presence of the L-arginine infusion, suggesting that the formation of NO was dependent on free L-arginine alone.

Because these data provided only circumstantial evidence for L-arginine being the precursor for the synthesis of NO, definitive experiments were carried out using mass spectrometry and [$^{15}$N]L-arginine which demonstrated the formation of $^{15}$NO from the terminal guanidino nitrogen atom(s) of L-arginine when the cells were stimulated with bradykinin (Palmer et al., 1988a; fig. 3). The formation of $NO_2^-$ from L-arginine by endothelial cells was reported by others (Schmidt et al., 1988b). The conversion of L-arginine to NO is specific because a number of analogues of L-arginine, including its D-enantiomer, are not substrates. Furthermore, it was found that the release of NO from endothelial cells in culture could be inhibited in an enantiomerically specific manner by L-NMMA (Palmer et al., 1988b), an inhibitor of the generation of $NO_2^-$ and $NO_3^-$ and citrulline from L-arginine in macrophages (Hibbs et al., 1987a).

Endothelial homogenates form citrulline from L-arginine by a mechanism that is NADPH dependent and inhibited by L-NMMA (Palmer and Moncada, 1989). In endothelial cell cytosol, depleted of L-arginine by anion-exchange chromatography, there was an L-arginine-dependent increase in cyclic GMP which was also concentration dependent, required NADPH, and was accompanied by the formation of [$^3$H]citrulline from [$^3$H]arginine (Moncada and Palmer, 1990). Both the production of [$^3$H]citrulline and the increases in cyclic GMP were inhibited by L- but not D-NMMA. All of these data are consistent with NO and citrulline being coproducts of the same enzymatic reaction. In addition, the formation of [$^3$H]citrulline and the increase in cyclic GMP were

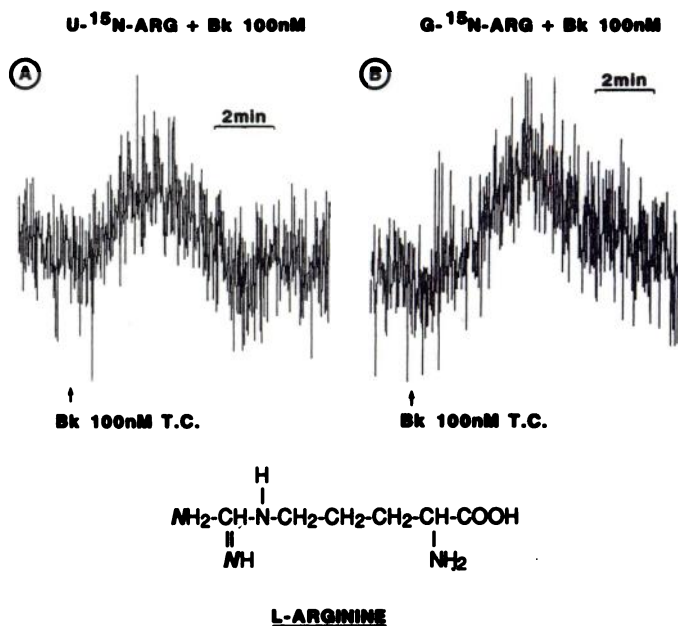U-$^{15}$N-ARG + Bk 100nM      G-$^{15}$N-ARG + Bk 100nM



FIG. 3. Determination of $^{15}$NO release from porcine aortic endothelial cells by mass spectrometry. A column packed with microcarriers covered with endothelial cells that had been cultured in medium without L-arginine (ARG) for 24 h was perfused with Krebs' buffer (5 ml/min). The effluent was passed continuously into the chemiluminescence reflux vessel, as described for fig. 1 except that NO was removed from the refluxing mixture under reduced pressure in a stream of He. The He stream was passed continuously into the source of an MS 50 TC (Kratos) mass spectrometer and $^{15}$NO ($m/z = 30.9950$) was determined at high resolution by electron impact mass spectrometry with single ion monitoring. The identity of the signal was confirmed by reference to the natural abundance of $^{15}$NO in NO standards which was found to be 0.37%. $^{15}$NO was released from cells stimulated with bradykinin (100 nM) in the presence of an infusion of U$^{15}$N-arginine (10 $\mu$M; A) or G$^{15}$N-arginine (10 $\mu$M; B). The cells released 2.2 ± 0.4 nmol $^{15}$NO ($n = 3$) in the presence of G$^{15}$N-arginine and 2.1 ± 0.7 nmol $^{15}$NO ($n = 4$) in the presence of U$^{15}$N-arginine, indicating that NO is derived from a guanidino nitrogen atom of L-arginine (shown as $N$). Reprinted with permission from Nature **333**: 664–666, 1988 (Macmillan Magazines Ltd.).

inhibited by $Ca^{2+}$ chelators, indicating that this enzyme, which has now been called NO synthase, is $Ca^{2+}$ dependent (Moncada and Palmer, 1990). Similar results have been reported by others (Mayer et al., 1989; Mulsch et al., 1989). Furthermore, NO synthesis from endothelial cell cytosol was inhibited by calmodulin-binding peptides and antagonists, an effect that was reversed by calmodulin, suggesting that the $Ca^{2+}$-dependent stimulation of NO synthase in endothelial cells is mediated by calmodulin (Busse and Mulsch, 1990a).

Recent evidence from studies using $^{18}O_2$ and mass spectrometry have shown that this enzyme incorporates molecular oxygen into both NO and citrulline, indicating that it is a dioxygenase (Leone et al., 1991).

*5. Inhibition of the synthesis of nitric oxide in the cardiovascular system.* a. IN VITRO. Data from experiments in vitro indicate that L-NMMA is a competitive inhibitor of the NO synthase (Palmer and Moncada, 1989; Mayer et al., 1989). An effect of this compound on

other arginine-metabolizing enzymes has not been reported, although it has been shown not to affect either arginase or arginine decarboxylase (Granger et al., 1990). L-NMMA also inhibits the release of NO from endothelial cells (Palmer et al., 1988b) and vascular tissues (Rees et al., 1989a; Amezcua et al., 1989). This compound has been a useful tool in the investigation of the biological significance of the L-arginine:NO pathway in the cardiovascular system.

L-NMMA induced an endothelium-dependent constriction of rabbit aortic rings, indicating that there is a continuous release of NO which maintains a dilator tone in this tissue (Palmer et al., 1988b). The removal of this basal tone accounts for all the constrictor activity of L-NMMA, including its apparent nonspecific endothelium-dependent vasoconstrictor effect on rat aortic rings (Thomas et al., 1989). Furthermore, L-NMMA inhibited endothelium-dependent relaxation induced by ACh, A23187, and substance P (Palmer et al., 1988b; Rees et al., 1989a). All of these effects could be reversed by L-arginine. Moreover, L-NMMA inhibited the release of NO induced by ACh from the perfused rabbit aorta, effects which were enantiomerically specific and reversible by L- but not D-arginine (Rees et al., 1989a). Inhibition by L-NMMA of histamine- and ACh-induced endothelium-dependent relaxation of the guinea pig pulmonary artery was also reversible by L-arginine (Sakuma et al., 1988). L-NMMA has since been used to examine the role of the L-arginine:NO pathway in the response to vasodilators in other vascular preparations in vitro, including rat aorta (Giuliani et al., 1990), canine cerebral arteries (Katusic et al., 1990), and human arteries and veins (Yang et al., 1990).

The importance of NO, formed from L-arginine, in regulating basal tone and the response to endothelium-dependent dilators in resistance vessels was demonstrated using L-NMMA in the isolated perfused rabbit heart (Amezcua et al., 1989). In this preparation, L-NMMA caused an increase in coronary perfusion pressure and an inhibition of the decrease in coronary perfusion pressure induced by ACh, accompanied by inhibition of the release of NO into the coronary effluent. These effects were enantiomerically specific and were attenuated by L- but not D-arginine. Interestingly, L-NMMA, by inducing what has been termed a transient, selective "biochemical denudation" of the preparation (Amezcua et al., 1989), revealed the direct vasoconstrictor action of ACh on smooth muscle. Some of these findings were subsequently observed in the isolated perfused guinea pig heart (Levi et al., 1990).

Recent evidence shows that removal of the endocardium induces a negative inotropic effect in isolated papillary muscle preparations (Smith et al., 1991). This, together with the finding that cultured porcine endocardial cells release NO and possess an NO synthase (Schulz et al., 1991), suggests that the L-arginine:NO pathway plays a role in myocardial contractility (Lewis et al., 1990; Smith et al., 1991).

b. IN VIVO. In anesthetized rabbits intravenous administration of L-NMMA, but not D-NMMA, induced an increase in blood pressure that could be reversed by L- but not D-arginine and was associated with a reduced release of NO from a perfused aortic segment obtained from treated animals (Rees et al., 1989b). This reduced release could be reversed by infusing L-arginine through the aortic segment in vitro. Thus, the basal release of NO derived from L-arginine plays an important role in regulating blood flow and pressure. Furthermore, the stimulated release of NO contributes to the response to endothelium-dependent vasodilators in vivo.

The hypotensive response to ACh in the anesthetized rabbit, rat, or guinea pig was inhibited only partially by L-NMMA (Rees et al., 1989b; 1990c; Whittle et al., 1989; Aisaka et al., 1990), and this effect was mainly detectable when expressed in terms of both the decrease and the duration of the hypotensive response. The reasons for this partial inhibition are not clear at present. Further work is required to clarify the mechanisms underlying these observations.

L-NMMA has subsequently been shown to cause an increase in blood pressure in anesthetized guinea pigs (Aisaka et al., 1989, 1990) and rats (Whittle et al., 1989; Gardiner et al., 1990a,b; Tolins and Raij, 1990; Tolins et al., 1990; Rees et al., 1990c). In one of these studies (Tolins et al., 1990), the vasodilation induced by ACh was accompanied by an increase in the urinary excretion of cyclic GMP, both of which were prevented by L-NMMA. Furthermore, the effects of L-NMMA on blood pressure were accompanied by a decrease in glomerular filtration rate.

The increase in blood pressure induced by L-NMMA was accompanied by a decrease in vascular conductance in the renal, mesenteric, carotid, and hindquarters vascular beds of conscious, chronically instrumented rats (Gardiner et al., 1990b). Furthermore, these effects were sustained if the infusion of L-NMMA was continued for 6 h (Gardiner et al., 1990c), indicating not only the critical role of NO in maintaining a dilator tone in all these beds but also the fact that regulatory systems in the vasculature are unable to reaccommodate the flow toward pretreatment levels. In awake, chronically instrumented dogs, L-NMMA induced a dose-related, L-arginine-reversible, constriction of the coronary circulation and a reduction in resting phasic coronary flow (Chu et al., 1991; fig. 4). The coronary vasodilation which follows vagal stimulation has also been suggested to be NO dependent (Broten et al., 1991).

Studies in which L-NMMA was infused in humans into the brachial artery or the dorsal veins of the hand demonstrated that the vasodilation induced by ACh or bradykinin, but not that induced by GTN, could be attenuated by this compound (Vallance et al., 1989a,b). Furthermore, whereas in the brachial artery L-NMMA induced direct vasoconstriction, it had no such direct
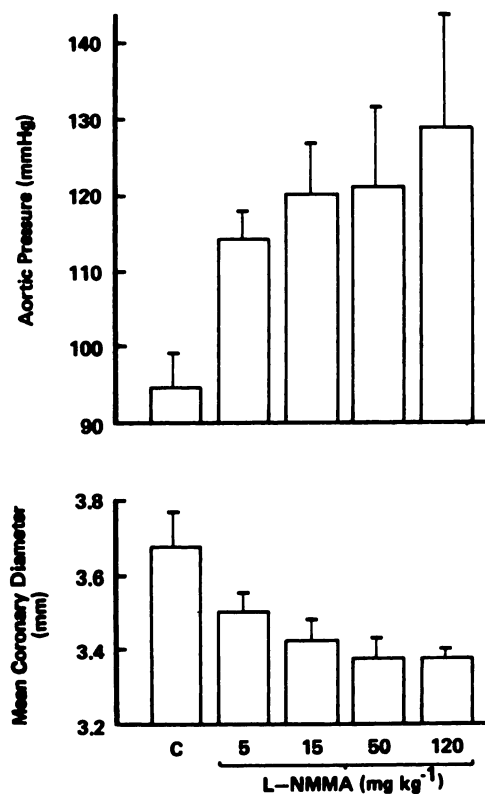
FIG. 4. Effect of L-NMMA on aortic pressure and mean coronary diameter in awake dogs. Infusion of L-NMMA (5 to 120 mg/kg) caused a dose-dependent increase in aortic pressure and a reduction in mean coronary diameter. C, control value before treatment with L-NMMA. Data from Chu et al., 1991.

effect on the hand veins. This suggests that on the arterial side of the circulation, but not the venous side, there is a continuous release of NO that maintains a dilator tone. That the arterial side of the circulation releases, in general, more NO than the venous side is also suggested by the fact that ACh-induced dilation in veins was rapidly transformed into constriction as the dose increased. Interestingly, the dilation was attenuated by L-NMMA, whereas the constriction was enhanced, suggesting that NO mediates, at least in part, the dilation and functionally antagonizes vasoconstrictor responses (Vallance et al., 1989b).

In the microcirculation of the hamster cheek pouch, intravenous L-NMMA caused a reduction in diameter of the arterioles but not the venules (Rees et al., 1990b). The arterioles of this preparation dilated in response to NO and to bradykinin (Rees et al., 1990b; Rivers et al., 1990). In the microcirculation of the rabbit skeletal muscle, however, L-NMMA constricted arterioles and, to a lesser extent, venules (Persson et al., 1990). Some inhibition of ACh-induced relaxation was observed in this preparation after administration of L-NMMA. In the rat skeletal muscle microcirculation, vasodilation induced by ACh, bradykinin, or adenosine triphosphate was blocked by methylene blue or hydroquinone (Kaley et al., 1989). In this preparation, however, A23187-induced vasodilation could also be inhibited by indomethacin

(Kaley et al., 1989), suggesting that NO is not the sole contributor to the action of some vasoactive agents known to release NO in other preparations. In the rat gastric microcirculation, L-NMMA, but not D-NMMA, reduced mucosal blood flow and, thus, compromised mucosal integrity. The effect of L-NMMA was reversed by L-arginine (Pique et al., 1989; Whittle et al., 1990).

c. NOVEL INHIBITORS OF THE SYNTHESIS OF NITRIC OXIDE. Other L-arginine analogues have been described as inhibitors of NO generation in vascular tissue (fig. 5). An early study showing that prolonged incubation with L-canavanine inhibited endothelium-dependent relaxation in rings of rat aorta (Schmidt et al., 1988a) has recently been shown not to be due to inhibition of the endothelial NO synthase (Palmer and Moncada, 1989; Mayer et al., 1989). However, L-NA is an inhibitor of this enzyme (Rees et al., 1990c,d; Mulsch and Busse, 1990) and has effects on vascular tissue in vitro similar to those described for L-NMMA (Moore et al., 1989; Rees et al., 1990c,d; Ishii et al., 1990; Mulsch and Busse, 1990). Furthermore, L-NIO, L-NA, and its methyl ester L-NAME induced enantiomerically specific effects on vascular tissue in vivo and in vitro which were qualitatively similar to those described for L-NMMA (Rees et al., 1990c,d). L-NMMA, L-NIO, and L-NAME all inhibited the endothelial NO synthase, although L-NIO was approximately 5 times more potent than the other analogues. The different potency shown by these compounds in vascular tissue in vitro and in vivo (Rees et al., 1990c,d) may also be due to differences in uptake, distri-
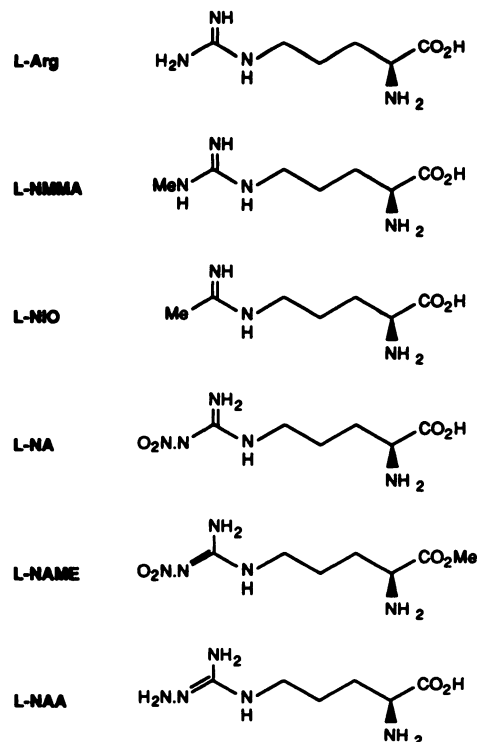
FIG. 5. Structural formulae of L-arginine and analogues that inhibit NO formation. L-Arg, L-arginine; L-NAA, $N^G$-amino-L-arginine.

bution, or metabolism of the compounds. Two of these compounds, L-NMMA and L-NAME, are orally active, because they induced an increase in blood pressure when given by this route to Brattleboro rats (Gardiner et al., 1990d; fig. 6).

Recently, another L-arginine analogue, $N^G$-amino-L-arginine, has been shown to be an inhibitor of ACh-induced relaxation and cyclic GMP accumulation in isolated rings of bovine pulmonary artery (Fukuto et al., 1990). This compound also inhibited NO synthesis by endothelial cells and caused a pressor response in the anesthetized guinea pig (Gross et al., 1990).

*6. Physiological implications.* The implications of the existence of an NO-mediated vasodilator tone are many. The present concepts of the mechanics of the vascular tree consider it either as a system of resistance vessels against which the heart pumps the blood or as a resistance system modulated by neural or hormonal vasoconstrictor and vasodilator influences.

The physiological stimuli for generation of NO are not yet fully understood, but pulsatile flow and shear stress seem to be two of the main determinants (Pohl et al., 1986a; Rubanyi et al., 1986). Endothelium-dependent flow-induced dilation has been described in conduit arteries in vitro and in vivo (Hintze and Vatner, 1984; Hull et al., 1986; Pohl et al., 1986b; Miller and Vanhoutte, 1988). Chronic increases in blood flow caused by an arteriovenous fistula in the hindlimb of the dog have been shown to augment endothelium-dependent relaxation to ACh (Miller and Vanhoutte, 1988). Moreover, Griffith et al. (1987) reported that, in resistance arteries of the intact rabbit ear, endothelium-dependent vasodilation in response to ACh was enhanced at increased flows. These authors subsequently showed that the activity of endogenous NO was greatest in large arterioles in which hydraulic resistance and shear stress were also highest (Griffith and Edwards, 1990).

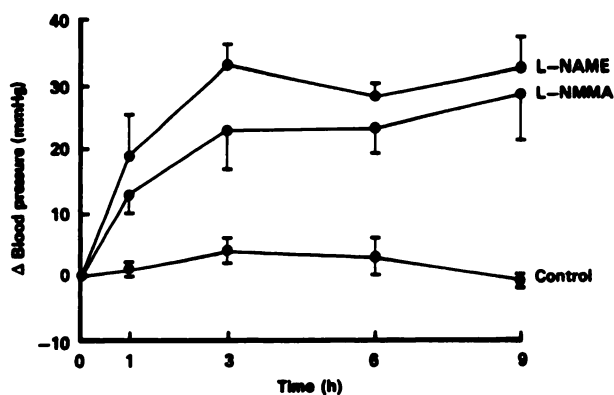Flow-induced dilation in canine epicardial arteries fol-



FIG. 6. Changes in blood pressure induced by oral administration of L-NAME and L-NMMA. Brattleboro (genetically vasopressin-deficient) rats were given L-NAME (0.1 mg/ml) or L-NMMA (1 mg/ml) in their drinking water for 9 h (*n* = 8 for each). Both compounds induced an increase in blood pressure which persisted for the duration of the experiment. Data from Gardiner et al., 1990d.

lowing reactive hyperemia was markedly attenuated after endothelial denudation of the vessel (Inoue et al., 1988). In addition, NO was released from endothelial cells in culture in a flow-dependent manner (Kelm et al., 1991). Moreover, although the bradykinin-induced release of NO from perfused guinea pig hearts increased at enhanced flow rates, it was not dependent on changes in perfusion pressure (Kelm et al., 1991). Flow-dependent coronary dilation has also been demonstrated in humans (Drexler et al., 1989). The role of other factors such as sympathetic or parasympathetic innervation in the physiological generation of NO or whether some endothelial cells release vasoactive substances, which in turn induce the release of NO, has not yet been elucidated (Parnavelas et al., 1985; De Nucci et al., 1988; Hopwood et al., 1989; Warner et al., 1989).

It is likely that NO-dependent vasodilator tone is entirely locally regulated and, as such, is probably one of the simplest and yet most fundamental adaptive mechanisms in the cardiovascular system.

*7. Pharmacological implications.* The available evidence, therefore, indicates that the cardiovascular system is in a state of constant active vasodilation dependent on the generation of NO. Indeed, NO can now be considered the endogenous nitrovasodilator (Moncada et al., 1988a).

Nitrovasodilators have been used clinically for about 100 years and are still widely used in conditions such as angina pectoris, congestive heart failure, hypertensive emergencies, pulmonary hypertension, fibrinolysis, percutaneous coronary angioplasty, and complications after cardiac catheterization (Abrams, 1987). Little was known about the mode of action of this class of compound. The vasodilator action of GTN was earlier believed to be due to its conversion in the circulation to $NO_2^-$ which, in contrast to $NO_3^-$, has some vasodilator action. However, in 1940 Krantz and coworkers demonstrated that an immediate and total conversion in the bloodstream of an effective vasodilator dose of GTN would not yield sufficient $NO_2^-$ to explain the observed vasodilation.

In 1977, two groups demonstrated independently that organic nitrates induced a dose-dependent increase in the levels of cyclic GMP in smooth muscle (Schultz et al., 1977; Katsuki et al., 1977). Subsequently, biochemical experiments showed that all of the nitrovasodilators and NO activate the soluble guanylate cyclase (Murad et al., 1978; Kukovetz et al., 1979). It is now widely accepted that activation by the nitrovasodilators of this enzyme and the consequent increase in cyclic GMP levels induces a sequence of protein phosphorylation associated with smooth muscle relaxation.

The precise way in which nitrovasodilators activate the soluble guanylate cyclase has been the subject of much debate. It has been suggested that free radicals, including NO, are the actual activators of the soluble guanylate cyclase (Katsuki et al., 1977). An alternative

hypothesis was proposed (Ignarro et al., 1981) whereby $NO_2^-$, released by metabolism of the nitrovasodilators, reacts with thiol groups to form S-nitrosothiols, which in turn activate the soluble guanylate cyclase. It has recently been shown, however, that nitrovasodilators also generate NO in a nonenzymic reaction with cysteine (Feelisch and Noack, 1987) and that the stimulation of the soluble guanylate cyclase by these compounds is dependent on the generation of NO (Feelisch and Noack, 1987; Feelisch et al., 1988). Furthermore, the concentrations of the nitrovasodilators that induced half-maximal stimulation of guanylate cyclase induced uniform release of NO in this reaction (Feelisch and Noack, 1987).

Some nitrovasodilators, such as SNP, release NO spontaneously, others such as the organic nitrates require the prior interaction with a thiol like cysteine, and the sydnonimines, such as 3,morpholino-sydnonimine, release NO subsequent to base-catalysed hydrolysis (for review, see Feelisch and Noack, 1991). These data, considered together, strongly suggest that NO is the final common effector molecule of all nitrovasodilators that activates the soluble guanylate cyclase.

The existence of an NO-dependent vasodilator tone has also led to the demonstration that the removal of such a tone, with the consequent up-regulation of the receptor for NO, the soluble guanylate cyclase, results in an increased sensitivity to those vasodilators that act by stimulating this enzyme (Moncada et al., 1991). Increased sensitivity to such vasodilators, which has been demonstrated in vitro following removal of the endothelium (Shirasaki and Su, 1985) or after treatment with nonspecific (Alheid et al., 1987) or specific inhibitors of the synthesis of NO (Luscher et al., 1989; Flavahan and Vanhoutte, 1989; Busse et al., 1989), had been attributed to several different mechanisms. However, it has now been shown that this increase in sensitivity to nitrovasodilators also occurs in vivo (fig. 7) and is accompanied in vitro by an increased formation of cyclic GMP in response to these vasodilators (Moncada et al., 1991).

Thus, removal of the basal NO tone in the cardiovascular system produces a phenomenon akin to denervation supersensitivity in which the mechanical or chemical prevention of the release of a neurotransmitter leads to an increased sensitivity to the actions of this neurotransmitter applied exogenously (Cannon and Rosenblueth, 1949). Whether after several days of treatment with an inhibitor of the NO synthase a more chronic type of supersensitivity develops, as a consequence of de novo synthesis of soluble guanylate cyclase, remains to be established. If this does occur, then the supersensitivity to exogenous NO would resemble denervation supersensitivity in both its acute and chronic characteristics.

In this context, it is interesting that venous tissue (Seidel and La Rochelle, 1987) and the venous circulation of humans in vivo (Vallance et al., 1989b) seem to have a lower basal release of NO and an increased sensitivity to nitrovasodilators when compared to the arterial side of the circulation. It is possible that this increased venous sensitivity is either the result of a soluble guanylate cyclase which is more sensitive to stimulation by exogenous NO or that the venous smooth muscle contains more soluble guanylate cyclase.

The concept of altered vascular reactivity following changes in the basal NO tone may also explain some aspects of the tolerance to nitrovasodilators, which has been recognized since the last century (Laws, 1898). Although tolerance to GTN is due in part to an impairment in its metabolism to NO (Ignarro et al., 1981; Kukovetz and Holtzmann, 1983), a more general form of tolerance to nitrovasodilators is due to desensitization of the soluble guanylate cyclase to the action of NO (Axelsson and Anderson, 1983; Waldman et al., 1986). Indeed, this tolerance is the opposite of supersensitivity and is, therefore, similar to the postjunctional subsensitivity that follows an increase in the neurotransmitter (Fleming et al., 1973). As a result of this it is possible that long-term treatment with nitrovasodilators may downregulate the soluble guanylate cyclase or even the synthesis of NO. Indeed, chronic treatment of rabbits with molsidomine has been shown to result in reduced release of NO from the thoracic aorta in response to ACh (Bult et al., 1990b).

8. Pathological implications. The endogenous NO-dependent dilator tone and the concept of altered vascular sensitivity to nitrovasodilators after its removal may also be significant for the understanding of some pathophysiological observations. Furthermore, because of the phenomenon of supersensitivity, pathological conditions in which there is a decrease in the synthesis or release of NO should be associated with an enhanced responsiveness to nitrovasodilators unless accompanied by a defect in the effector mechanisms beyond the soluble guanylate cyclase.

It is possible that the loss of the NO-mediated vasodilator tone is at least as important in essential hypertension or in vasospastic phenomena as some of the vasoconstrictor factors previously recognized. In this context, dietary supplementation with calcium has been shown to reduce blood pressure in animals and humans with hypertension (Sowers et al., 1989) and in women with pregnancy-induced hypertension (Lopez-Jaramillo et al., 1989). The basal and stimulated release of NO from perfused aortic segments of the rabbit is sensitive to changes in the $Ca^{2+}$ concentration within the physiological range (Lopez-Jaramillo et al., 1990). Thus, in the vessel wall, a paradoxical situation may exist whereby the $Ca^{2+}$-dependent modulation of the release of NO from the endothelium may be antagonized by $Ca^{2+}$ acting at the level of the smooth muscle to favour contraction.

The vasodilator response to ACh infusion into the brachial artery has been shown to be reduced in patients with essential hypertension (Linder et al., 1990; Panza
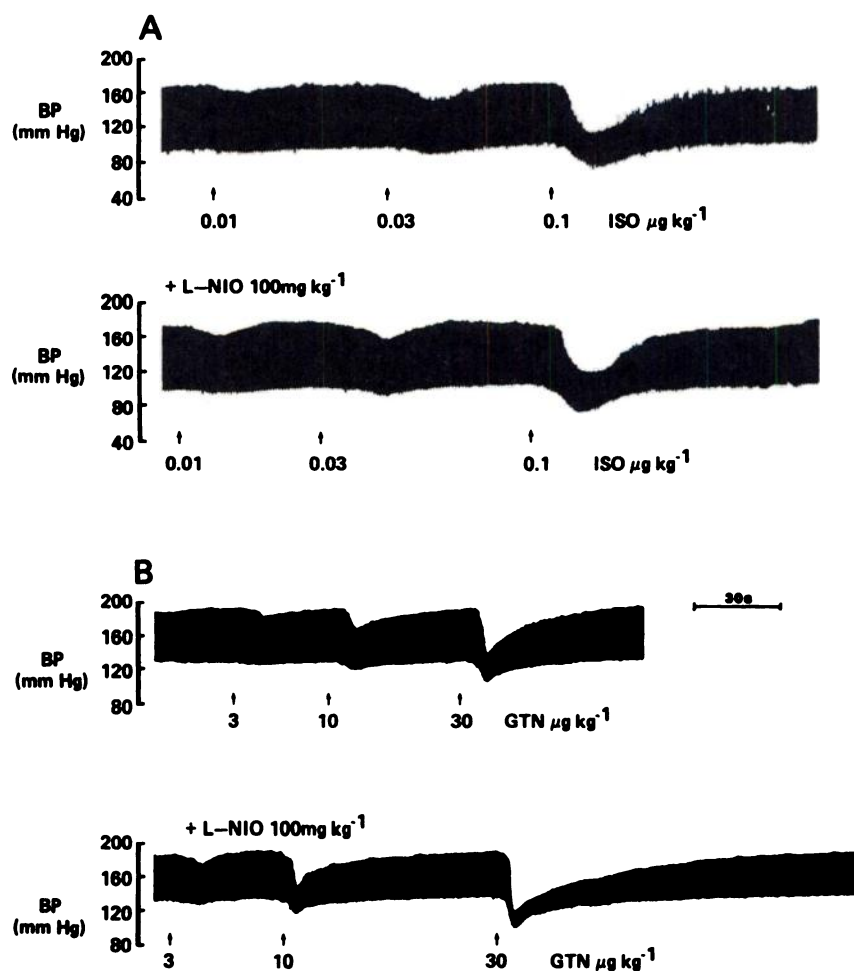
FIG. 7. Effect of L-NIO (100 mg/kg) on the hypotension induced in anesthetized rats by (A) isoprenaline (ISO, 0.01 to 0.1 μg/kg) and (B) GTN (3 to 30 μg/kg). The hypotensive responses to these compounds were compared under conditions in which initial mean arterial blood pressure (BP) was similarly elevated by a continuous infusion of phenylephrine or by a bolus dose of L-NIO in the same animal. The hypotensive response to GTN, but not to ISO, was significantly potentiated by L-NIO. Data from Moncada et al., 1991 and reprinted with permission.

et al., 1990), whereas the response to SNP was unchanged or enhanced in such patients. Impaired endothelium-dependent relaxation has also been observed in blood vessels of genetically diabetic rats (Durante et al., 1988; Kappagoda et al., 1989) and by some (Kamata et al., 1989) but not other (Kappagoda et al., 1989) workers using blood vessels from rats with drug-induced diabetes.

In animal models of hypertension, several groups have reported reduced endothelium-dependent vascular responses (Winquist et al., 1984; De Mey and Gray, 1985; Luscher et al., 1987; Otsuka et al., 1988), in some cases accompanied by reduced levels of cyclic GMP (Otsuka et al., 1988). Interestingly, the changes in both these parameters could be reversed by restoring the blood pressure to normal (Luscher et al., 1987; Otsuka et al., 1988). A strong correlation has been shown between the impairment of endothelium-dependent relaxation and the level and duration of hypertension in different strains of spontaneously hypertensive rats (Sunano et al., 1989). Other workers, however, have reported no change (Hagen and Webb, 1984) or an increase (Konishi and Su, 1983) in endothelium-dependent relaxation in arteries from hypertensive animals. All of these experiments are not strictly comparable, because different vascular preparations from different species stimulated with different agonists were used. Thus, there is a need for a systematic study of the effects of endothelium-dependent vasodilators in hypertension.

A reduction in the release of EDRF from the vascular endothelium or a decrease in endothelium-dependent relaxation has been demonstrated in vascular tissue obtained from rabbits made atherosclerotic (Coene et al., 1985; Verbeuren et al., 1986; Sreeharan et al., 1986; Henry et al., 1987; Guerra et al., 1989) and in human atherosclerotic coronary arteries (Forstermann, 1986). Endothelial damage also induced an increase in basal tone and enhanced responses to vasoconstrictor substances in the coronary arteries of the atherosclerotic miniature swine (Shimokawa et al., 1983). Furthermore, the response of the coronary circulation to ACh in humans is decreased in patients with coronary artery disease (Drexler et al., 1989), and arteries obtained from

hearts of such patients show a reduced basal and stimulated release of NO (Chester et al., 1990).

The reasons for these changes need to be elucidated; however, it is possible that they may be related to the generation of $O_2^-$ released by monocytes that accumulate during the atherosclerotic process, since oxygen-derived radicals and lipid peroxides have been implicated in the development of atherosclerosis. Another possibility is that oxidized low-density lipoproteins (a known risk factor in coronary artery disease) could contribute to the endothelial dysfunction found in atherosclerotic arteries, for they have been shown to inhibit endothelium-dependent relaxation (Jacobs et al., 1990).

Inhibition of NO by Hb could play a role in the vasospasm that follows subarachnoid hemorrhage, which has long been suspected to be mediated by some product of lysed red blood cells (Fisher et al., 1981). A recent study of the canine basilar artery in vitro showed that endothelium-dependent relaxation induced by A23187 was inhibited by Hb and by cerebrospinal fluid obtained from patients with subarachnoid hemorrhage (Kanamaru et al., 1987). Furthermore, intracisternal injections of Hb in the pig induce concentration-dependent vasoconstriction in intrathecal arteries (Byrne et al., 1989). Because NO is also an inhibitor of platelet activation, impairment of its formation in the vessel wall will not only predispose to vasoconstriction but also favor platelet adhesion, aggregation, and the consequent release of vasoconstrictor substances that will exacerbate the tendency to vasospasm. In this context, platelet products released during aggregation in vitro contracted deendothelialized canine coronary artery rings, whereas they induced relaxation when the endothelium was present (Houston et al., 1985). Moreover, reduced NO production could lead to enhanced adhesion of platelets to the vessel wall, as has been observed in conditions such as atherosclerosis and diabetes, whereas lack of NO could be involved in the restenosis sometimes seen after angioplasty. Interestingly, isosorbide dinitrate, which releases NO, and prostaglandin $E_1$ acted synergistically to reduce platelet deposition and to increase their survival time in patients with peripheral vascular disease (Sinzinger et al., 1990).

A decreased synthesis of NO may contribute in other ways to the origin of conditions such as atherosclerosis and hypertension, e.g., endogenous NO has been shown to inhibit the release of renin (Vidal et al., 1988). NO-releasing vasodilators inhibit proliferation of vascular smooth muscle cells by a cyclic GMP-mediated process (Garg and Hassid, 1989). Furthermore, NO has been reported to inhibit mitogen release from stimulated human platelets (Barrett et al., 1989), and SNP prolongs fibrinolysis, possibly by preventing the release from platelets of an inhibitor of tissue plasminogen activator (Korbut et al., 1990).

Finally, the discovery of the biochemical pathway for the formation of NO in the vessel wall and its role as the endogenous nitrovasodilator will accelerate the search for novel compounds that either imitate its action or increase its production in the cardiovascular system.

### B. Platelets

1. *Pharmacological actions of nitric oxide.* EDRF, which was first defined in terms of its vascular relaxant activity, was later found also to inhibit platelet aggregation (Azuma et al., 1986; Furlong et al., 1987; Radomski et al., 1987a). NO inhibited platelet aggregation via a cyclic GMP-dependent mechanism (Mellion et al., 1981; Radomski et al., 1987a). Prostacyclin and NO acted synergistically to inhibit aggregation and to disaggregate platelets (Radomski et al., 1987b), suggesting that the release of NO and prostacyclin by the vascular endothelium plays a role in its thromboresistant properties. Furthermore, like prostacyclin, NO also has a cytoprotective effect (Radomski et al., 1988). Thus, the interaction of these two compounds might prove to have a superior antithrombotic effect to that of either of them alone (Radomski et al., 1987b; Macdonald et al., 1988; Sinzinger et al., 1990).

NO also inhibited platelet adhesion to collagen fibrils, endothelial cell matrix, and endothelial cell monolayers (Radomski et al., 1987c,d), a process mediated via cyclic GMP. Prostacyclin has a weak inhibitory effect on platelet adhesion (Higgs et al., 1978) and this, in contrast to its effect on aggregation that is cyclic AMP dependent, is also mediated by cyclic GMP (Radomski et al., 1987c). This suggests that cyclic GMP regulates adhesion, whereas both cyclic AMP and cyclic GMP regulate aggregation. Prostacyclin did not synergize with NO to prevent platelet adhesion (Radomski et al., 1987c), indicating that the physiological process of platelet adhesion and repair of the vessel wall may proceed under circumstances in which both NO and prostacyclin are acting together to exert a powerful antithrombotic action.

Carbachol, an endothelium-dependent vasodilator, when given intravenously to rabbits, caused an inhibition of ex vivo platelet aggregation and elevation of platelet cyclic GMP (Hogan et al., 1988). In rats, carbachol has been shown to inhibit ADP-induced accumulation of indium 111-labeled platelets in the pulmonary circulation (Bhardwaj et al., 1988). The effect of nitrovasodilators on platelet aggregation has been poorly understood, because SNP inhibits aggregation, whereas GTN is almost inactive. However, only those nitrovasodilators that spontaneously release NO, such as SNP and 3,morpholino-sydnonimine, inhibit platelet aggregation (Gerzer et al., 1988), suggesting that platelets lack the mechanisms required for the uptake and/or conversion of organic nitrates to NO.

2. *Synthesis of nitric oxide.* Aggregation of platelets is accompanied by an increase in their cyclic GMP levels (Bohme et al., 1974; Haslam et al., 1978; Steer and

Salzman, 1980). This was originally thought to mediate aggregation (Chiang et al., 1976; Glass et al., 1977) by opposing the actions of cyclic AMP (Marcus and Zucker, 1965; Mills and Smith, 1971). However, the finding in 1981 that nitrovasodilators and NO, which elevate cyclic GMP levels, concomitantly inhibit aggregation led to the suggestion that increased cyclic GMP levels may, in fact, be related to inhibition of platelet aggregation (Mellion et al., 1981).

Recently, it has become clear that platelets also generate NO and that the L-arginine:NO pathway acts as a negative feedback mechanism to regulate platelet aggregation (Radomski et al., 1990a). Aggregation induced by collagen was accompanied by an increase in intraplatelet levels of cyclic GMP but not cyclic AMP. L-NMMA inhibited this increase in cyclic GMP and enhanced aggregation. Furthermore, L-arginine, which had no effect on basal levels of cyclic GMP, enhanced the increase in cyclic GMP induced by collagen and inhibited aggregation (fig. 8). An increase in cyclic GMP in platelet cytosol was observed not only with SNP but also with L-arginine and, to a lesser extent, with L-homoarginine. The effect of L-arginine was enantiomer specific, inhibited by L-NMMA, and dependent on the presence of NADPH. In addition, measurements in the platelet cytosol demonstrated an L-arginine- and NADPH-dependent formation of NO (fig. 8) which was inhibited by L-NMMA, providing conclusive evidence for the existence of the L-arginine:NO pathway in the platelets.

The formation of NO from L-arginine in platelet cytosol was dependent on the free $Ca^{2+}$ concentration, showing that the NO synthase in platelets is $Ca^{2+}$ de-

pendent. Other similarities with the endothelial NO synthase were that the platelet enzyme did not require a cofactor other than NADPH, L-homoarginine was a weak substrate, and the enzyme was not inhibited by L-canavanine (Radomski et al., 1990a,b).

Interestingly, L-arginine did not increase the basal levels of cyclic GMP in unstimulated platelets but did so only when they were stimulated with collagen. This shows that this NO synthase can utilize exogenous substrate after it is activated. Whether this is a consequence of the uptake of arginine into the cell or of the regulation of the NO synthase by the substrate is not yet known. In this context it is worth noting that L-arginine was 20 to 30 times less active as an inhibitor of platelet aggregation in blood than in washed platelets. This may be a reflection of a down-regulation of L-arginine uptake into the platelets by the high concentrations (48 to 140 $\mu$M) of L-arginine in blood (Matsumoto et al., 1976; Glass et al., 1986).

Other aggregating agents, such as ADP and arachidonic acid, also stimulated the synthesis of NO by platelets. Moreover, aggregation induced by these agents was inhibited by L- but not D-arginine (Radomski et al., 1990b). In contrast, the aggregation induced by A23187 and thrombin could only be inhibited by L-arginine in conditions in which the effect of NO would be potentiated, such as in the presence of an inhibitor of cyclic GMP phosphodiesterase or of a subthreshold concentration of prostacyclin. The inhibitors of NO synthase L-NMMA, L-NAME, and L-NIO were similarly effective in potentiating aggregation induced by arachidonic acid and ADP but were less so for thrombin and were ineffec-
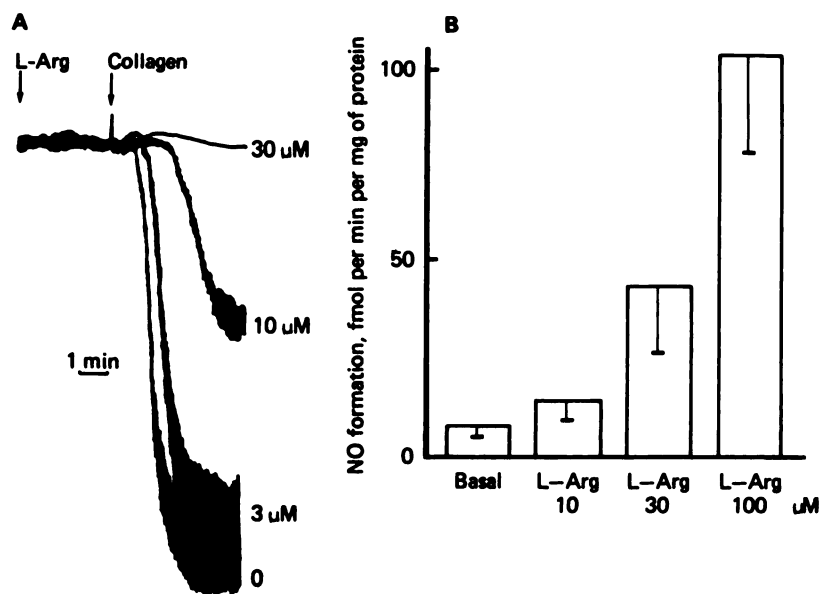


FIG. 8. Effect of L-arginine on platelet aggregation and on NO synthesis in platelet cytosol. A, Aggregation of human platelets induced by collagen (1 $\mu$g/ml) was measured as change in light transmission. L-Arginine (L-arg; 3 to 30 $\mu$M) inhibited aggregation in a concentration-dependent manner ($n = 5$). B, Basal rate of NO formation in platelet cytosol, measured spectrophotometrically in the presence of NADPH, was enhanced in a concentration-dependent manner by L-arginine ($n = 3$). Data from Radomski et al., 1990a and reprinted with permission.

tive against aggregation induced by A23187 (Radomski et al., 1990b). This profile of modulation of platelet aggregation by endogenous NO correlates well with that observed for the rank order of potency of exogenous NO against the aggregating action of these agents (collagen = arachidonic acid = ADP > thrombin > A23187; Radomski et al., 1987a).

The L-arginine analogues L-NMMA, L-NAME, and L-NIO potentiated collagen-induced platelet aggregation to similar extents and with similar potency. However, L-NIO was approximately 8 times more potent than L-NMMA and L-NAME as an inhibitor of NO synthase in platelet cytosol (Radomski et al., 1990b). This is similar to the order of potency of these compounds against endothelial NO synthase (Rees et al., 1990c). The reason for the difference in potency of these compounds in whole platelets and platelet cytosol is not known but may indicate differences in the uptake, distribution, or metabolism of these compounds in the platelets.

Hb did not reverse the effects of L-arginine in washed platelets. However, it was the most effective inhibitor of the increase in cyclic GMP induced by L-arginine in platelet cytosol which shows, as expected, that Hb did not penetrate the platelet membrane efficiently (Radomski et al., 1990b) and also that the formation of NO in the platelets acts as an autocrine regulatory system.

The stimulation of platelets with aggregating agents results in an approximately 100-fold increase in the resting intracellular $Ca^{2+}$ concentration (Ware et al., 1986), and it is likely that this increase activates the NO synthase. The mechanism by which elevated cyclic GMP levels attenuate platelet aggregation is not known; however, the increases in cyclic GMP cause $Ca^{2+}$ sequestration (Busse et al., 1987), leaving less free $Ca^{2+}$ available for aggregation. The net biological effect after stimulation of the platelets will, therefore, depend on the amounts of free $Ca^{2+}$, for it appears that, even when NO synthase is fully active, agents that are more powerful inducers of increases in intracellular $Ca^{2+}$ concentration, such as A23187, overcome the inhibitory effect of intraplatelet NO.

Thus, in the platelet the L-arginine:NO pathway acts as an autocrine negative feedback mechanism to regulate reactivity. Platelet aggregation in vivo is also likely to be regulated by intraplatelet NO, as well as by NO and prostacyclin released from vascular endothelium. The combined action of these two mediators could result in a synergistic suppression of intracellular $Ca^{2+}$ elevation and inhibition of platelet aggregation (Radomski et al., 1987b).

C. Nervous System

1. Central nervous system. Neurotransmission by agents such as ACh, glutamate, and glycine has long been known to be associated with elevated cyclic GMP levels in the brain and particularly in the cerebellum (for review, see Garthwaite, 1990). In 1974, glutamate was shown to cause cyclic GMP accumulation in cerebellar slices, a process that required $Ca^{2+}$ (Ferrendelli et al., 1974). Cerebellar cell suspensions containing a mixture of cell types, but not a preparation of highly purified Purkinje cells, also showed glutamate-induced accumulation of cyclic GMP (Garthwaite, 1990).

In 1977 NO was shown to stimulate the soluble guanylate cyclase in homogenates of mouse cerebral cortex (Miki et al., 1977). In the same year, the soluble fraction of rat forebrain was shown to contain a low molecular weight substance that activated soluble guanylate cyclase and whose action was inhibited by Hb (Deguchi, 1977). This activator was thought to be related to the nitrosamines or to act at the same allosteric site (Deguchi et al., 1978). Similar findings were reported in the rat cerebellum (Yoshikawa and Kuriyama, 1980). In 1982 the endogenous activator of the soluble guanylate cyclase in neuroblastoma cells was identified as L-arginine (Deguchi and Yoshioka, 1982). These observations, together with the discovery of the L-arginine:NO pathway in the vascular endothelium, led to the investigation of the existence of this pathway in the central nervous system.

Addition of L-arginine to rat synaptosomal cytosol in the presence of NADPH resulted in the formation of NO and citrulline and was accompanied by stimulation of soluble guanylate cyclase (Knowles et al., 1989). Both of these processes were inhibited by Hb and by L-NMMA but not by L-canavanine. These data showed that the rat brain possesses the NO synthase. This enzyme was dependent on the free $Ca^{2+}$ concentration and was virtually inactive at the resting free $Ca^{2+}$ concentration in synaptosomes (approximately 80 nM; Ashley et al., 1984), whereas it was fully active at $Ca^{2+}$ concentrations of approximately 400 nM (Knowles et al., 1989; fig. 9). As in the vascular endothelium and the platelet, therefore, increases in intracellular $Ca^{2+}$ may constitute the physiological mechanism for stimulating the synthesis of NO. Furthermore, like the enzyme in the vascular endothelium, it also incorporates $^{18}O_2$ into citrulline (Leone et al., 1991).

Stimulation of rat cerebellar cells with NMDA had been shown to induce an elevation of cyclic GMP levels which was associated with the release of an EDRF-like material (Garthwaite et al., 1988). Moreover, the cells that released this EDRF-like material in response to NMDA were not the target cells in which cyclic GMP levels were elevated. The cyclic GMP responses to NMDA (Garthwaite et al., 1989a; Bredt and Snyder, 1989) and to kainate (Garthwaite et al., 1989b) stimulation in rat cerebellar slices was later shown to be enhanced by L-arginine and inhibited by L-NMMA, in a manner that was reversed by L-arginine, showing that this response was indeed mediated by NO. More recently, it has been shown that L-NMMA administered intracerebellarly in mice inhibits the increase in cyclic GMP induced by NMDA, quisqualate, kainate, harmaline, and
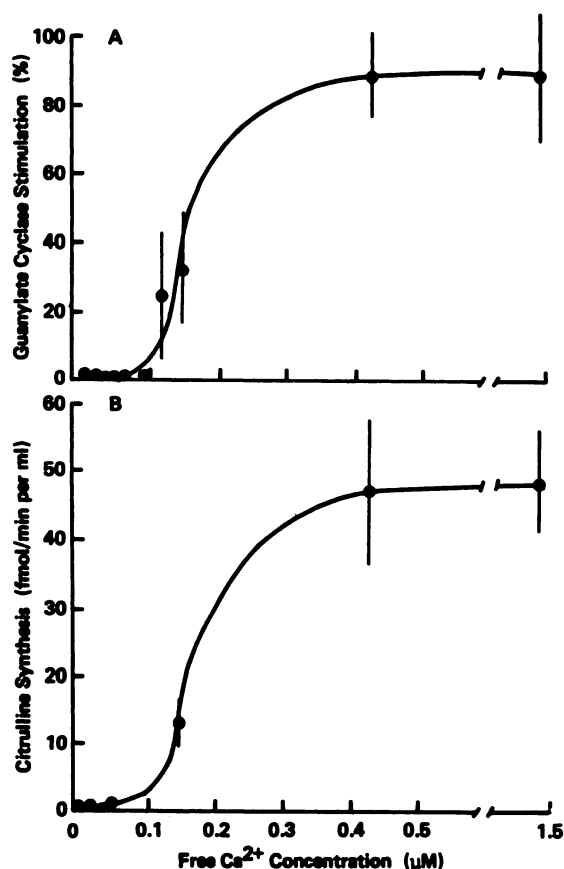
FIG. 9. $Ca^{2+}$ dependence of NO and citrulline synthesis from L-arginine by rat brain synaptosomal cytosol. The rates of synthesis of NO from L-arginine (100 $\mu M$) at various free $Ca^{2+}$ concentrations was determined by stimulation of guanylate cyclase ($A$) or by the synthesis of $^3$[H]citrulline from 0.2 $\mu M$ L-[$^3$H]arginine ($B$). Data from Knowles et al., 1989 and reprinted with permission.

pentylenetetrazole (Wood et al., 1990a), showing that the L-arginine:NO pathway mediates the increases in cyclic GMP induced by these compounds in vivo.

Interestingly, physiological $Ca^{2+}$ levels, which are essential for the action of the NO synthase, were found to inhibit the brain soluble guanylate cyclase (Olson et al., 1976; Knowles et al., 1989). This could represent a control mechanism whereby guanylate cyclase is not activated in those cells stimulated to produce NO but only in the effector cells (Knowles et al., 1989).

The NO synthase in the brain has been characterized and shown to be inhibited competitively by L-NMMA, L-NA, and L-NIO (Knowles et al., 1990b). Like the endothelial cell and platelet enzyme, the brain NO synthase only required NADPH as a cofactor. Bovine brain cytosol has also been shown to contain the NO synthase (Schmidt et al., 1989b). This enzyme has now been purified from rat cerebellum and shown to be calmodulin dependent (Bredt and Snyder, 1990). The purified enzyme migrates as a single 150 kDa band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and appears to be a monomer.

Cytosolic preparations of different brain regions

showed that the highest concentration of NO synthase was present in the cerebellum, followed by the hypothalamus and midbrain, striatum, and hippocampus, with the lowest activity found in the medulla oblongata (Forstermann et al., 1990). NO has also been found to be released from astrocytes following stimulation with bradykinin and A23187 (Murphy et al., 1990). The granule cells have been suggested to be the principal neurons in the cerebellum which release NO in response to exogenous NMDA receptor agonists in this tissue (Garthwaite and Garthwaite, 1987; Garthwaite, 1990). Histochemical studies using antibodies to the NO synthase have shown it to occur widely in the central nervous system (Ross et al., 1990), primarily in neurons and also in the vascular endothelium, with no glial localization. However, the specific localization of the enzyme in the cerebellum (Ross et al., 1990; Bredt et al., 1990) and other regions of the central nervous system needs further investigation.

The different mediator systems for which the L-arginine:NO pathway acts as a transduction mechanism to stimulate the soluble guanylate cyclase in the brain have not been established. As far as the biological function of NO in the brain is concerned, it is possible that it plays a role in the short-term effects of excitatory amino acids as well as in their long-term effects on brain development, learning, and memory. Furthermore, this pathway may mediate the biological effects of other neurotransmitters whose actions are associated with increases in cyclic GMP (Drummond, 1984).

The L-arginine:NO pathway may also play a role in the pathology of the central nervous system (see Moncada et al., 1989). The $Ca^{2+}$ influx that accompanies prolonged NMDA receptor activation is associated with degeneration of the neurons (Rothman and Olney, 1987; Choi, 1988; Garthwaite, 1989). It is likely that excessive NMDA receptor activation, with the consequent increase in $Ca^{2+}$, contributes to glutamate neurotoxicity by enhanced production of NO.

High levels of cyclic GMP also cause destruction of photoreceptor cells in the retina, where NO synthase has now been demonstrated by immunocytochemistry (Ross et al., 1990). This effect may explain the pathogenesis of some inherited retinal diseases in animals (Lolley et al., 1977). Cyclic GMP has also been suggested to have a role in seizures, because the levels of this nucleotide increase in several brain regions prior to the onset of drug-induced convulsions (Ferrendelli et al., 1980). Moreover, superfusion of cyclic GMP analogues onto grafts of hippocampus triggers prolonged epileptiform activity in the pyramidal neurons (Freedman et al., 1979). It is, therefore, important to note that antagonists of excitatory amino acid receptors or inhibitors of glutamate release have both antiepileptic actions and also protect the brain against ischemic damage which is thought to be mediated by excessive release of glutamate (Meldrum, 1990).

2. *Peripheral nervous system.* Nerves whose transmitter is neither ACh nor noradrenaline (i.e., NANC) form an important component of the autonomic innervation of smooth muscle in the gastrointestinal tract, the pelvic viscera, the airways, and other systems (Burnstock, 1979; Gillespie, 1982; Barnes, 1986; Gillespie et al., 1990). The nature of the neurotransmitter released by these nerves has been the subject of much debate. Purines such as adenosine or ATP, neuropeptides such as vasoactive intestinal peptide or substance P, and the inhibitory factor extracted from bovine retractor penis muscle have all been proposed as possible candidates. Recently, however, evidence has been accumulating to indicate that NO may be the transmitter in at least some of these nerves and may be a modulator in others.

The rat anococcygeus muscle has a motor noradrenergic and an inhibitory NANC innervation (Gillespie, 1972). When the noradrenergic nerves were blocked with guanethidine, field stimulation of this preparation produced a rapidly developing and powerful relaxation. This response was mimicked by nitrovasodilators, including NO (for review, see Gillespie et al., 1990), was blocked by Hb (Bowman and Gillespie, 1982) and anoxia (Bowman and McGrath, 1985) and was $Ca^{2+}$ dependent (De Luca et al., 1991). Recently, L-NMMA was shown to inhibit NANC-mediated relaxation of the rat anococcygeus muscle, without affecting the response to SNP (Li and Rand, 1989). This effect was reversed or prevented by L-arginine. Similar effects were observed by Gillespie et al. (1989), who also noted that L-NMMA caused an increase in tone in this tissue, which was reversible by L-arginine. In the mouse anococcygeus muscle NANC relaxations were blocked by both L-NMMA and L-NA, with the latter being 30 to 50 times more potent than L-NMMA (Gibson et al., 1989, 1990). In both the rat and mouse anococcygeus preparations, the contractile response to noradrenergic stimulation was enhanced in the presence of L-NMMA or L-NA (Li and Rand, 1989; Gibson et al., 1990).

Stimulation of NANC nerves in the canine ileocolonic junction has recently been shown to release a factor whose biological actions, chemical instability, inactivation by $O_2^-$ and Hb, inhibition by L-NA, and potentiation by L-arginine all indicate it to be NO (Bult et al., 1990a). Thus, NO can be considered the biological mediator of some NANC neurotransmission.

A possible neuromodulatory role for NO has been proposed in other systems. L-NMMA enhanced nerve-induced contractions in the guinea pig ileum and pulmonary artery (Gustafsson et al., 1990) and in dog mesenteric artery segments (Toda and Okamura, 1990). Furthermore, it suppressed the relaxant response to transmural nerve stimulation in the dog cerebral artery strips (Toda and Okamura, 1990) and partially inhibited the relaxant response to transmural stimulation in the guinea pig taenia coli (Gustafsson et al., 1990). Further

study is required to ascertain whether NO modulates the release or action of a vasodilator transmitter released from nerves or whether it acts as a transmitter from these nerves to the effector smooth muscle.

The peripheral analgesic effect of ACh has also been proposed to be mediated via NO (Duarte et al., 1990). ACh, L-arginine, and SNP induced analgesia in an animal model, and L-NMMA prevented the analgesia induced by both ACh and L-arginine. Furthermore, analgesia induced by ACh was potentiated by an inhibitor of cyclic GMP phosphodiesterase. Whether this is the result of NO generation in the nociceptors or in cells closely associated with them and whether the L-arginine:NO pathway controls the input of nociceptive information into the nervous system requires investigation.

### D. Other Cells and Tissues

An NO synthase in the adrenal gland has been identified and partially characterized (Palacios et al., 1989). Cyclic GMP levels in the soluble fraction of homogenates of rat adrenal glands were stimulated by SNP and S-nitroso-N-acetyl penicillamine, known activators of soluble guanylate cyclase, demonstrating the presence of this enzyme in this preparation. The soluble guanylate cyclase in both the cortex and medulla was also stimulated in the presence of L-arginine, and this stimulation was inhibited by Hb. Furthermore, the stimulation by L-arginine was inhibited by L-NMMA, L-NA, L-NAME, L-NIO, and L-canavanine and was associated with the formation of both $NO_2^-$ and citrulline. Thus, an L-arginine:NO pathway is present in both the cortex and the medulla of the adrenal gland. This NO synthase is also NADPH and $Ca^{2+}$ dependent, like that in the vascular endothelium, the platelet, and the brain.

The functional importance of this pathway in regulating adrenal cortex and medulla function is not clear. A role for cyclic GMP has been implicated in both catecholamine secretion (Derome et al., 1981; Dohi et al., 1983; O'Sullivan and Burgoyne, 1990) and steroidogenesis (Perchellet et al., 1978). It is, therefore, possible that the L-arginine:NO pathway plays a modulatory role in the control of the synthesis and secretion of hormones by the adrenal gland.

Evidence for the presence of the L-arginine:NO pathway has also been found in some cell lines including mouse neuroblastoma cells (Ishii et al., 1989; Gorsky et al., 1990) and porcine kidney epithelial cells stimulated with oxytocin (Ishii et al., 1989) or with vasopressin (Schroder and Schror, 1989). Recently, rat mast cells have also been shown to produce an NO-like substance that modulates the release of histamine (Salvemini et al., 1990).

### III. Nitric Oxide as an Effector Molecule in Immunological Reactions

### A. Macrophages

1. *Synthesis of nitric oxide.* The link between N-nitroso compounds and an increased risk of cancer, together

with the demonstration that such compounds could be produced in vivo as a result of a reaction between ingested secondary amines and $NO_2^-$, prompted investigation into the metabolism of inorganic nitrogen oxides in humans (for review, see Leaf et al., 1990b).

When humans were fed a low $NO_3^-$ diet, endogenous synthesis of $NO_3^-$ was observed (Green et al., 1981b). This process, which was estimated to produce approximately 1 mmol $NO_3^-$/day in humans under normal conditions (Wagner et al., 1983a), was originally believed to be a result of intestinal microbial metabolism but was later shown, in germ-free animals, to be mammalian in origin (Green et al., 1981a). A marked increase in urinary $NO_3^-$ excretion was observed in humans with diarrhea and fever (Hegesh and Shiloah, 1982; Wagner et al., 1984). Furthermore, treatment of rats with *Escherichia coli* LPS led to an increase in the urinary output of $NO_3^-$ which correlated with the degree of fever (Wagner et al., 1983b). Turpentine and carrageenin produced similar, although smaller, increases in urinary $NO_3^-$ in rats. These results suggested a correlation between immuno-stimulation and elevated $NO_3^-$ synthesis. Later work by Stuehr and Marletta (1985) showed that blood levels and urinary excretion of $NO_3^-$ increased after exposure to LPS in LPS-sensitive, but not in LPS-resistant, mice and that activated mouse peritoneal macrophages showed increased $NO_2^-$ and $NO_3^-$ production in vitro. Thus, the macrophage seems to be the most likely source of LPS-induced $NO_2^-$ and $NO_3^-$ synthesis.

Murine peritoneal macrophages in culture, activated with LPS, synthesized approximately 150 nmol $NO_3^-$/ $10^6$ cells/24 h (Stuehr and Marletta, 1987a). Other immunostimulants, such as Bacillus Calmette-Guerin, also increase $NO_3^-$ production in animals. Moreover, macrophage cell lines also generated $NO_3^-$ in response to IFN-γ, either alone or in combination with LPS or with TNF (Stuehr and Marletta, 1987a,b; Ding et al., 1988). The macrophage cell line, RAW 264.7, formed $NO_2^-$ and $NO_3^-$ after activation with LPS and IFN-γ. This was dependent on the presence of L-arginine, which was shown by ^{14}C-labeling studies to be converted to citrulline (Iyengar et al., 1987). Experiments with ^{15}N-labeled arginine showed that both $NO_2^-$ and $NO_3^-$ were derived from the terminal guanidino nitrogen atom(s) of this amino acid (Iyengar et al., 1987). Although it had previously been supposed that the $NO_3^-$ resulted from the oxidation of $NO_2^-$, it was now apparent that both were derived from a common intermediate.

At about the same time, the cytotoxicity of activated macrophages against tumour target cells was shown to be dependent on the presence of L-arginine (Hibbs et al., 1987b). These authors also showed that activated macrophages synthesized L-citrulline and $NO_2^-$ from L-arginine and that L-NMMA prevented the synthesis of both of these products as well as the expression of cytotoxicity (Hibbs et al., 1987b).

Following the demonstration of the synthesis of NO from L-arginine by endothelial cells (Palmer et al., 1987, 1988a), it became apparent that NO was the most likely inorganic nitrogen oxide intermediate in the pathway of $NO_2^-$ and $NO_3^-$ synthesis in the macrophage. Three groups subsequently demonstrated that NO synthesized from L-arginine was indeed the precursor of the $NO_2^-$ and $NO_3^-$ in these cells (Marletta et al., 1988; Hibbs et al., 1988; Stuehr et al., 1989).

*2. Characteristics of the nitric oxide synthase.* The NO synthase in the macrophage differs from that in the endothelial cell, platelet, and brain in that it was not detectable in either macrophage cell lines or freshly elicited macrophages that had not been activated by an agent such as LPS, alone or in combination with IFN-γ (Stuehr and Marletta, 1985, 1987a,b), and it required protein synthesis for its expression (Marletta et al., 1988). There was a lag phase of approximately 8 h before $NO_2^-$ and $NO_3^-$ synthesis was detected. The synthesis of these products then continued either until no more substrate was available or until the death of the cell (Stuehr and Marletta, 1987a).

When macrophages activated with LPS and IFN-γ were cultured in medium without free amino acids, L-arginine was found to be the only amino acid essential for cytotoxicity (Hibbs et al., 1987b) and for $NO_2^-$ and $NO_3^-$ synthesis (Iyengar et al., 1987). L-Homoarginine, L-arginine methyl ester, L-arginamide, and L-arginyl-L-aspartate could also substitute for L-arginine (Hibbs et al., 1987b; Iyengar et al., 1987). The reaction was enantiomer specific, because D-arginine was not a substrate. Furthermore, neither $NH_3$ nor hydroxylamine was a substrate, ruling out the possibility that L-arginine was hydrolyzed to citrulline and $NH_3$ which was then oxidized to $NO_2^-$ and $NO_3^-$. Formation of $NO_2^-$ and $NO_3^-$ in the macrophage was inhibited not only by L-NMMA but also by L-canavanine (Iyengar et al., 1987). This sensitivity to L-canavanine is an additional difference between the NO synthase in the macrophage and that in the endothelial cell, platelet, and brain.

More recently, L-NIO has been shown to be a potent, fast-acting, and irreversible inhibitor of NO generation in the macrophage cell line J774 activated with IFN-γ and LPS (McCall et al., 1991a; fig. 10). In contrast, the actions of L-NMMA were found to be slower in onset, and L-NAME and L-NA were considerably less potent and their effects were fully reversible (Gross et al., 1990; McCall et al., 1991a). These properties make L-NIO a potentially useful tool to investigate the generation of NO in phagocytic cells.

Studies of the NO synthase in LPS/IFN-γ-activated RAW 264.7 cells showed that the enzymatic activity, which was not present in nonactivated cells, was cytosolic (Marletta et al., 1988). The enzyme required L-arginine and NADPH and its activity was enhanced by $Mg^{2+}$, although this cation was not essential for the
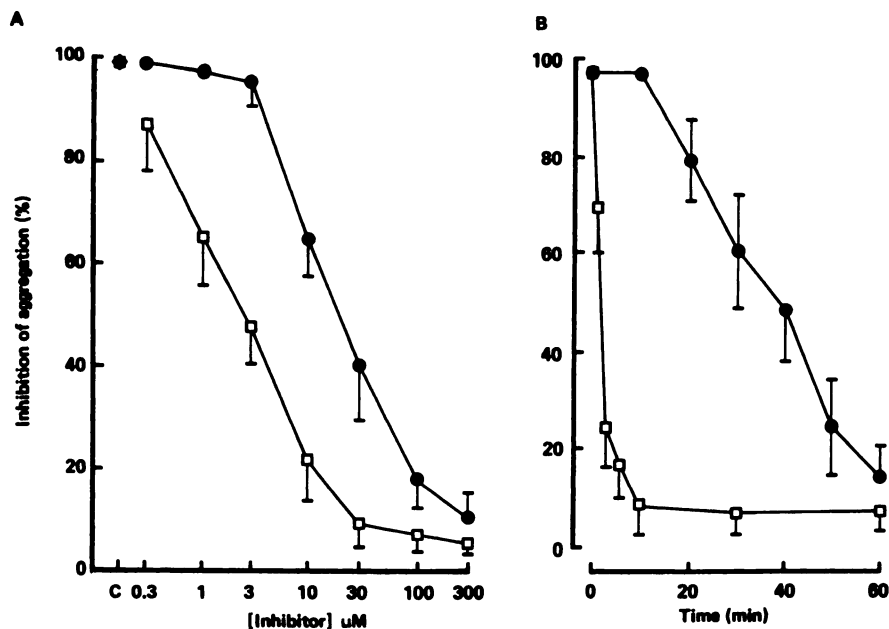
FIG. 10. Effect of L-NIO and L-NMMA on platelet anti-aggregatory activity of the macrophage cell line J774. *A*, Inhibition of platelet aggregation by $1 \times 10^6$ activated (IFN-$\gamma$ + LPS for 18 h) J774 cells (C, *) is prevented in a dose-dependent manner by treatment for 10 min with L-NIO (□) or for 50 min with L-NMMA (●). *B*, Time course of the effect of L-NIO (□) and L-NMMA (●) on the anti-aggregatory activity of J774 cells. Both compounds were studied at 100 $\mu$M. Data from McCall et al., 1991 and used with permission.

formation of NO. The cytosol of activated J774 cells has also been shown to contain an inducible NO synthase which is NADPH dependent and does not require $Ca^{2+}$ (McCall et al., 1991a). NO generation from activated macrophage cytosol has also been shown to be dependent on the presence of tetrahydrobiopterin (Tayeh and Marletta, 1989; Kwon et al., 1989) and to be stimulated by flavin adenine dinucleotide and reduced glutathione (Stuehr et al., 1990).

Evidence from studies using $^{18}O_2$ and mass spectrometry has shown that the inducible NO synthase in activated RAW 264.7 cells incorporates molecular oxygen into citrulline (Kwon et al., 1990). In addition, molecular oxygen is incorporated into both NO and citrulline by the inducible NO synthase from activated J774 cells (Leone et al., 1991). This shows that the inducible NO synthase, like the constitutive enzyme, can be classified as a dioxygenase.

The similarities and differences between the two NO synthases are shown in table 1.

*3. Physiology and pathophysiology of the synthesis of nitric oxide.* Macrophages are activated by cytokines from sensitized lymphocytes responding to specific antigen (Mackaness, 1964, 1969). The activated macrophages subsequently express nonspecific cytotoxic activity against microorganisms such as bacteria or protozoa (Remington and Merigan, 1969). Similarly, microorganisms or LPS treatment can modify the behaviour of macrophages in such a way that they become cytotoxic for neoplastic cells (Old et al., 1959). This activated macrophage cytotoxic effect is not dependent on phagocytosis and can be cytostatic and in some cases cytolytic (for review, see Hibbs et al., 1990).

Cocultivation of activated macrophages with tumour cells or microorganisms resulted in inhibition of DNA replication in the target cell (Keller, 1973; Krahenbuhl and Remington, 1974; Granger et al., 1980). Furthermore, target cell mitochondrial respiration was prevented as a result of inhibition of complex I and complex II of the mitochondrial electron transport system (Granger et al.,

TABLE 1
*Similarities and differences between the two NO synthases*

| Constitutive | Inducible |
|---|---|
| Cytosolic* | Cytosolic |
| NADPH dependent | NADPH dependent |
| Dioxygenase | Dioxygenase |
| Inhibited by L-arginine analogues | Inhibited by L-arginine analogues |
| $Ca^{2+}$/calmodulin dependent | $Ca^{2+}$/calmodulin independent |
| Picomoles NO released | Nanomoles NO released |
| Short-lasting release | Long-lasting release |
| Unaffected by glucocorticoids | Induction inhibited by glucocorticoids |

* Evidence for a particulate form of this enzyme in the vascular endothelium has recently been reported (Forstermann et al., 1991).

1980; Granger and Lehninger, 1982). Aconitase, a citric acid cycle enzyme, was also inhibited in target cells (Kilbourn et al., 1984). Inhibition of complex I, complex II, and aconitase has also been observed in the activated macrophages themselves (Drapier and Hibbs, 1988). Treatment of guinea pig hepatoma cells with NO under anaerobic conditions caused intracellular iron loss, inhibition of mitochondrial respiration and of aconitase and DNA synthesis, and cytostasis (Hibbs et al., 1988). Similar results were later reported by Stuehr and Nathan (1989).

All of the enzymes affected by the activated macrophages, including ribonucleotide reductase (the rate-limiting enzyme in DNA replication), contain catalytically active nonhaem iron coordinated to sulphur atoms (Hibbs et al., 1990). The inhibition of enzyme activity was accompanied by the release of intracellular iron from target cells (Hibbs et al., 1984). Exposure of Fe-S groups to NO results in iron-nitrosyl complex formation (Salerno et al., 1976; Reddy et al., 1983). L-Arginine-dependent nitrosyl-iron-sulphur complex formation in macrophages activated by a cell-mediated immune response in vivo or by IFN-γ in vitro, but not in nonactivated macrophages, has been demonstrated (Lancaster and Hibbs, 1990; Pellat et al., 1990). A signal characteristic of paramagnetic nitrosyl-iron-sulphur complexes in activated macrophages has been observed using electron paramagnetic resonance spectroscopy (Pellat et al., 1990). This signal was similar to that described by Vanin (1967) following treatment of biological materials containing iron-sulphur complexes with NO. Thus, these results provide a molecular mechanism for the way in which the L-arginine:NO pathway brings about activated macrophage-induced cytotoxicity.

It is important to stress that NO is in some cells cytotoxic and in others cytostatic (Hibbs et al., 1990), suggesting that the sensitivity to NO varies from one cell to another. The reasons for this are not yet clear, but they may be dependent on the relative importance of iron- sulphur-centered enzymes in different cells.

Interestingly, it was known as long ago as 1977 that macrophage-induced cytotoxicity could be inhibited by the presence of Hb or erythrocytes (Weinberg and Hibbs, 1977). Furthermore, there have been reports since the 1950s of the inhibitory effect of L-arginine on the growth of experimental tumours (Levy et al., 1954; Weisburger et al., 1969; Takeda et al., 1975; Cho-Chung et al., 1981).

It is also possible that other actions of NO contribute to the cytotoxic effects of activated macrophages. Cyclic nucleotides are involved in the regulation of several macrophage functions, including phagocytosis, motility, responses to lymphokines, and DNA synthesis. Macrophages treated with NO-generating agents showed an increase in cyclic GMP levels (Bromberg and Pick, 1980) and NO generated by macrophage cytosol stimulates soluble guanylate cyclase (Mulsch et al., 1990). These data suggest that macrophage cytotoxicity may also be related to the formation of cyclic GMP. NO-generating compounds can also stimulate an ADP-ribosyltransferase in various tissues (Brune and Lapetina, 1989). The functional consequences of this action are not known.

The role of NO formed from L-arginine as an effector mechanism in macrophage cytotoxicity of a number of microorganisms has now been demonstrated. Macrophages activated with LPS and IFN-γ had a powerful cytostatic effect in vitro for the fungal pathogen Cryptococcus neoformans (Granger et al., 1986a,b). This fungistasis was dependent on the presence of L-arginine (Granger et al., 1986a), was inhibited by L-NMMA, and correlated with the synthesis of L-citrulline, $NO_2^-$, and $NO_3^-$ (Granger et al., 1990). Similarly, the cytostatic actions of macrophages against Toxoplasma gondii were prevented by L-NMMA (Adams et al., 1990). Intraperitoneal administration of killed Corynebacterium parvum in mice also led to increased resistance to infection with Toxoplasma, an effect that was also prevented by L-NMMA (Adams et al., 1990). Intracellular Leishmania major amastigotes were also destroyed by a mechanism that was inhibited by L-NMMA (Green et al., 1990; Liew et al., 1990; fig. 11). Moreover, this parasite was killed directly by exposure to NO (Liew et al., 1990). Mice infected with L. major developed larger lesions following in vivo treatment with L-NMMA. The infected footpads of the L-NMMA-treated mice contained $10^4$ times more parasites than those of control mice or of mice treated with D-NMMA (Liew et al., 1990). NO produced by activated macrophages may also be involved in the in vitro killing of Schistosoma mansoni (James and Claven, 1989).

The L-arginine:NO pathway has been proposed to be a primary defence mechanism against intracellular microorganisms as well as pathogens such as fungi and helminths that are too large to be phagocytosed (Hibbs et al., 1990). Interestingly, at wounds and other sites of inflammation, reduced L-arginine and increased ornithine concentrations occur as a result of the action of arginase derived from macrophages (Edlebacher and Merz, 1927; Currie et al., 1979; Albina et al., 1988). Arginase activity, which is also increased in tumour cells (Yamanaka et al., 1972; Redmond and Rothberg, 1978; Thomasset et al., 1982), promotes cellular proliferation and tissue regeneration by providing ornithine which is a substrate for polyamine and collagen synthesis (Haddox and Russell, 1981; Albina et al., 1988). Furthermore, the resulting low arginine concentrations (<0.1 mM; Albina et al., 1988) enhance macrophage activation-associated functions but reduce their cytotoxicity (Albina et al., 1989a). The interactions between these two pathways of L-arginine metabolism, leading to the formation of urea/ornithine or NO/citrulline, and their significance in macrophage biology require further investigation.
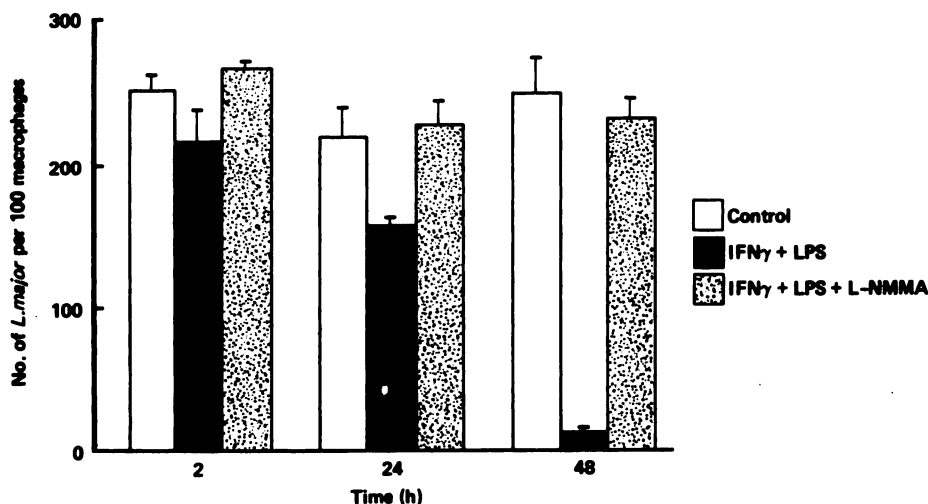
FIG. 11. Effect of L-NMMA on leishmanicidal activity of activated macrophages. Mouse peritoneal exudate cells were activated in vitro with IFN-γ (400 units/ml) and LPS (10 ng/ml) in the presence or absence of L-NMMA (500 μM) and were infected with *L. major* promastigotes. The number of intracellular amastigotes determined by visual counting at different times after infection did not change appreciably in macrophages cultured in medium alone (□). In contrast, the number of parasites in activated macrophages decreased progressively (■). Such leishmanicidal activity was completely inhibited by L-NMMA (■). Data from Liew et al., 1990.

## B. Neutrophils

Rat peritoneal neutrophils elicited with glycogen were found to release a factor that relaxed vascular smooth muscle (Rimele et al., 1988) and increased cyclic GMP levels in this tissue (Lee et al., 1988). Human neutrophils were subsequently shown to produce a substance that inhibited platelet aggregation, an effect accompanied by increased levels of cyclic GMP in the platelets (Salvemini et al., 1989). The production of this antiaggregating substance from rat peritoneal neutrophils was inhibited by L-NMMA (McCall et al., 1989). The identity of this EDRF-like factor released from stimulated human neutrophils was confirmed as NO using chemiluminescence (Schmidt et al., 1989a; Wright et al., 1989).

L-Canavanine, which inhibits the formation of NO in the macrophage, but not in the endothelial cell, also inhibited the release of NO from neutrophils (McCall et al., 1989; Schmidt et al., 1989a). The inhibitory effects of both L-canavanine and L-NMMA required a preincubation time of approximately 1 h, probably reflecting the slow uptake of these inhibitors into the neutrophils. As in the macrophage, the inhibitory effect of L-NMMA was reversed by concomitant incubation with L-arginine, L-homoarginine, or L-arginyl-L-aspartate, but not D-arginine (McCall et al., 1989).

The release of NO from rat peritoneal neutrophils could also be inhibited by L-NIO (McCall et al., 1990). Unlike the effect of L-NMMA and L-canavanine, the effect of L-NIO was rapid in onset, reaching full inhibitory effect after 10 min of incubation. Furthermore, the maximum degree of inhibition of NO formation observed with L-NIO was significantly greater than that with L-NMMA and was not reversed by L-arginine. Thus, the uptake of this compound into neutrophils differs significantly from that of L-NMMA and L-canavanine. This

suggests that the uptake of L-arginine analogues into cells is a process that may differ from one cell to another.

The presence of the NO synthase in neutrophils has recently been confirmed (McCall et al., 1991a). The enzyme has the same characteristics as that found in the J774 cells in that it is cytosolic, $Ca^{2+}$ independent, and inducible (McCall et al., 1991b). Interestingly, L-NAME, which is a potent inhibitor of endothelial NO synthesis (Rees et al., 1990c), did not affect it in the neutrophil (McCall et al., 1990, 1991a). However, both L-NA and L-NAME were weak inhibitors of NO synthase isolated from the neutrophil (McCall et al., 1991a).

The synthesis of NO by neutrophils was enhanced by stimulation with the chemoattractants FMLP and leukotriene $B_4$ (McCall et al., 1989). However, at higher concentrations of FMLP the release of $O_2^-$ could also be detected. This $O_2^-$ interacted with the NO such that the levels of biologically active NO decreased as the degree of stimulation and of $O_2^-$ production increased. Leukotriene $B_4$, unlike FMLP, released only small amounts of $O_2^-$ at the concentrations at which it stimulated NO release (McCall et al., 1989). Treatment of the neutrophils with SOD enhanced the biological action of NO released by FMLP, as did L-arginine (McCall et al., 1990). This shows that the same biological effect can be achieved either by scavenging $O_2^-$ (with SOD) or by increasing the synthesis of NO (with L-arginine).

L-Arginine did not affect the basal production of NO and only enhanced the release observed in the presence of FMLP (McCall et al., 1989). This suggests either that there was sufficient substrate for basal NO synthesis and this only became limiting during stimulation or that arginine was mobilized continuously and that this mobilization was not enhanced under conditions when the NO synthase is activated.

The demonstration of the interaction between $O_2^-$ and NO formed from L-arginine by neutrophils (McCall et al., 1989), which has also been shown when these cells are stimulated with phorbol myristate acetate (Sturm et al., 1989), may explain the differences in the amounts of NO released by neutrophils from different sources. Human neutrophils produce substantially more $O_2^-$ than do rat neutrophils and, therefore, the resultant amount of NO release detected in human neutrophils is low (McCall et al., 1990). This interaction may also compromise measurements of NO released by neutrophils in response to other stimuli by favouring $O_2^-$ release over enhancement of NO release (McCall et al., 1990; Rimele et al., 1990).

Recently, it has been suggested that NO and $O_2^-$ react rapidly to form the stable peroxynitrite anion, which decomposes when protonated into the potent oxidants OH• and $NO_2$• (Beckman et al., 1990). If this is the case, the protective action of SOD in ischaemic tissue may be due in part to the prevention of the formation of peroxynitrite and, hence, of these highly toxic radicals. However, at present there is no evidence for the occurrence of these reactions in vivo and therefore it remains to be established whether the interaction between NO and $O_2^-$ leads to an increased toxicity or is a neutralizing mechanism for two toxic radicals.

The biological significance of the production of NO by neutrophils remains to be elucidated. At present there is no evidence that neutrophil-derived NO plays a role in the cytotoxic activity of these cells. Data implicating NO in neutrophil function is inconsistent. For example, NO has been shown to inhibit neutrophil aggregation (McCall et al., 1988) and the NO-producing agents molsidomine and 3,morpholino-sydnonimine inhibit lysosomal enzyme release from neutrophils (Schroder et al., 1990). In contrast, it has been reported that inhibition of NO synthase by L-NMMA inhibits neutrophil chemotaxis (Kaplan et al., 1990).

## C. Kupffer Cells and Hepatocytes

Rat Kupffer cells cocultured with hepatocytes and stimulated with LPS induced a significant suppression of hepatocyte total protein synthesis but only when L-arginine was present in the medium (Billiar et al., 1989). This effect, which required an induction period of several hours, was associated with formation of $NO_2^-$, $NO_3^-$, and citrulline both in the Kupffer cells and the hepatocytes. These authors also showed that the supernatant from activated Kupffer cells induced the formation of NO in the hepatocytes, an effect that was blocked by L-NMMA (Curran et al., 1989). A function of this NO seems to be cytotoxicity because it suppresses hepatocyte protein synthesis (Curran et al., 1989).

## D. Vasculature

Until recently, it was considered that the only NO synthase in the vessel wall was the constitutive $Ca^{2+}$-dependent enzyme in the endothelium. However, porcine endothelial cells in culture have now been shown to express a $Ca^{2+}$-independent NO synthase following activation in vitro with LPS and IFN-$\gamma$ (Radomski et al., 1990c; fig. 12). Furthermore, endothelium-denuded rings incubated with LPS in vitro (Rees et al., 1990a) or obtained from rats treated with LPS (Knowles et al., 1990c) also exhibited $Ca^{2+}$-independent synthesis of NO. The induction of NO synthase in both the vascular endothelium and the vascular smooth muscle layer was time dependent and inhibited by cycloheximide, indicating that they were synthesized de novo and are similar to the NO synthase induced in other cells (Radomski et al., 1990c; Rees et al., 1990a). The induction by interleukin-1 of NO synthase in vascular smooth muscle cells in culture has also been demonstrated (Busse and Mulsch, 1990b; Beasley et al., 1991).

Rings of rat aorta, with or without endothelium, contracted with phenylephrine showed, after a 2-h lag period, a progressive decrease in tone over a period of 8 h (Rees et al., 1990a; fig. 13). This loss in tone was accompanied by increased levels of cyclic GMP, a decreased response to the constrictor effect of phenylephrine, and the ability of the tissues to relax to L-arginine, all of which could be prevented or reversed by inhibitors of NO synthase. These changes were associated with the appearance in the tissue of a $Ca^{2+}$-independent NO synthase. The induction of this enzyme and the accompanying functional and biochemical changes were prevented by cycloheximide and by polymyxin B, an antagonist of LPS, indicating that the LPS shown to be present in the experimental system was responsible for the induction of this enzyme (Rees et al., 1990a).
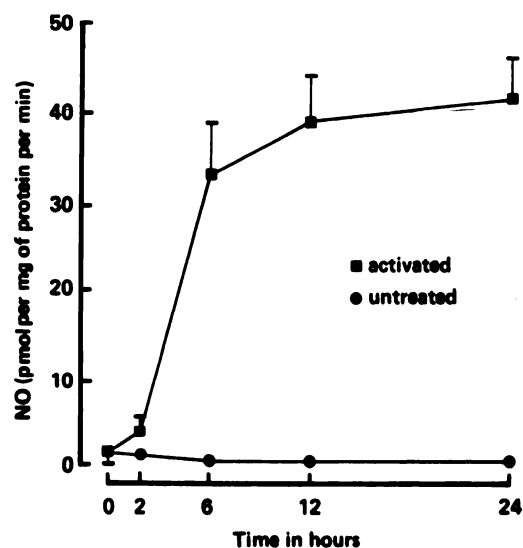


Fig. 12. Time course of induction of $Ca^{2+}$-independent NO synthase after activation of endothelial cells by LPS and IFN-$\gamma$. Porcine aortic endothelial cells, cultured on microcarrier beads, were incubated with LPS (10 $\mu$g/ml) and IFN-$\gamma$ (150 units/ml) for the time indicated and NO synthase activity was measured spectrophotometrically in the presence of ethyleneglycol bis($\beta$-aminoethyl ether)-N,N,N',N'-tetraacetic acid (1 mM). Data from Radomski et al., 1990c and reprinted with permission.

FIG. 13. The effect of cycloheximide and L-NIO on the time-dependent loss of tone in endothelium-denuded rings of rat aorta contracted with phenylephrine (40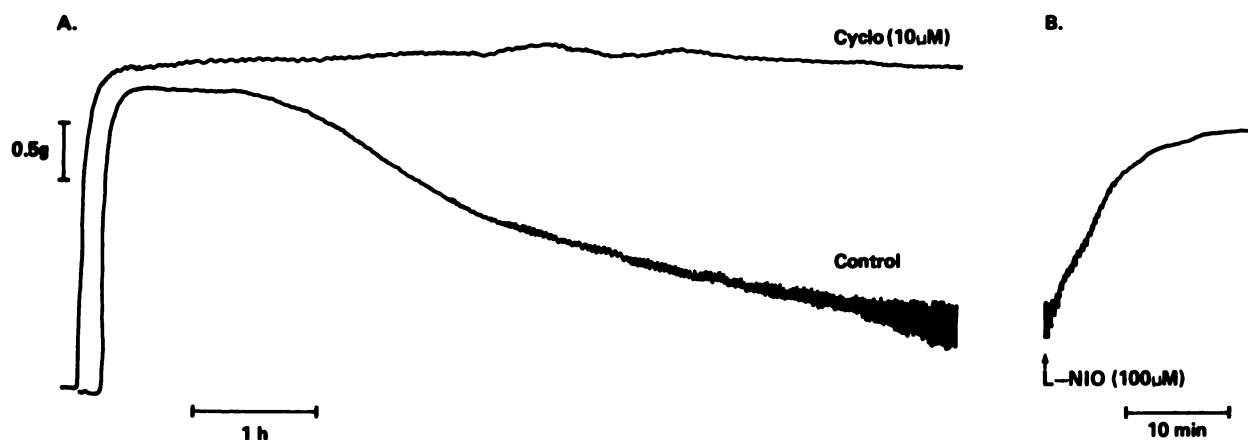 nM). A, Prolonged incubation of rat aortic rings in Krebs' buffer resulted in a time-dependent loss of tone which commenced after a lag period of approximately 2 h. When the rings were incubated throughout in the presence of cycloheximide (cyclo; 10 μM), no loss of tone was observed. B, Addition of L-NIO (100 μM) to the control rings, which showed time-dependent loss of tone, fully restored the tone to the initial level. Data from Rees et al., 1990a.

The induction of an NO synthase in the vessel wall explains some observations made over the years in vascular preparations, including the hyporeactivity to vasoconstrictors (Parratt, 1973; Wakabayashi et al., 1987; McKenna, 1990) and the elevations in cyclic GMP in smooth muscle after incubation with LPS (Fleming et al., 1990). Furthermore, rings of rat aorta or bovine pulmonary artery that did not relax to L-arginine when freshly isolated, did so after prolonged incubation in vitro. These observations, which were originally attributed to depletion of L-arginine in the smooth muscle (Schini et al., 1990; Wood et al., 1990b), can now be explained in terms of induction of an NO synthase in the vascular smooth muscle. Furthermore, the $Ca^{2+}$-independent, apparently constitutive enzyme described. in the endothelium (Mulsch et al., 1989) could be the result of induction of the NO synthase during the isolation of the cells.

### E. Other Cells and Tissues

An inducible NO synthase has also been found in EMT-6 cells, a spontaneous murine mammary adenocarcinoma cell line. When these cells were exposed to medium conditioned by activated macrophages they synthesized $NO_2^-$, $NO_3^-$, and L-citrulline (Amber et al., 1988). This synthesis was dependent on the presence of L-arginine and was accompanied by inhibition of aconitase and of DNA replication and the release of iron. Cycloheximide prevented all of these responses, showing that protein synthesis was required for the NO synthase to be induced (Amber et al., 1988). The same pattern of response could be induced in EMT-6 cells by IFN-γ in combination with LPS, TNF, or interleukin-1, where it was associated with prevention of cell proliferation (Lepoivre et al., 1989). Treatment of EMT-6 cells with IFN-γ and LPS stopped not only their own proliferation but also inhibited DNA synthesis in mouse, rat, and human

tumour cell lines cocultured with these cells (Lepoivre et al., 1990).

Murine fibroblasts, stimulated with cytokines, have also been shown to generate NO (Werner-Felmayer et al., 1990; J. B. Hibbs, Jr., personal communication). Because of the variety of the lineage of cells that have now been shown to respond to appropriate cytokines by expressing this NO synthase, one can conclude that this is not only a property of the reticuloendothelial system but is a general host defence response that is likely to be found in many other cells and tissues.

### F. Immunologically Induced Formation of Nitric Oxide in Vivo

Treatment of rats for 7 days with C. parvum induced NO synthase in hepatocytes; this correlated with an increase in blood levels of $NO_2^-$ and $NO_3^-$ which could be inhibited with L-NMMA (Billiar et al., 1990b). Furthermore, treatment of rats with LPS induced after 6 h an NO synthase in the lung and liver (Knowles et al., 1990a; fig. 14). This NO synthase, which was not present in the tissues from untreated rats, was $Ca^+$ independent. Moreover, in the liver the highest activity of this enzyme was localized in the hepatocytes (Knowles et al., 1990a). The induction of the NO synthase in the liver was maximal at 6 h and declined toward control levels in the next 18 h.

The induction of a $Ca^{2+}$-independent NO synthase in the endothelial and smooth muscle layer of aortae from rats treated with LPS has also been demonstrated (Knowles et al., 1990c). Furthermore, the highest activity of the enzyme found was localized in the vascular smooth muscle layer. The formation of an NO derivative of Hb has been shown in rats during endotoxin-induced and hemorrhagic shock (Westenberger et al., 1990).

In a model of sepsis-related hepatic injury, L-NMMA was found to elevate serum levels of aspartate aminotransferase and lactate dehydrogenase, indicating in-
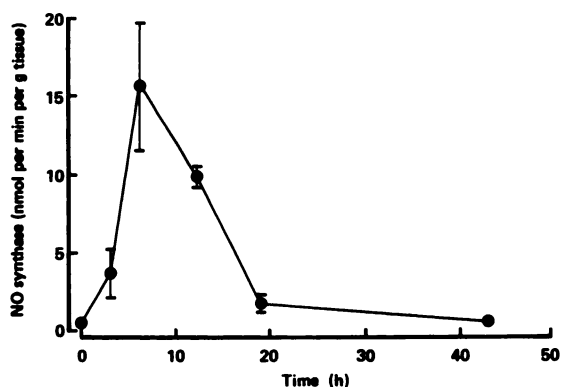
FIG. 14. NO synthase activity in rat liver following administration of LPS. In livers from untreated rats (0 h), there was no NO synthase activity detectable by spectrophotometry. However, treatment of the animals with LPS (2 mg/kg, i.p.) caused induction of an NO synthase, with maximal activity at 6 h. Data from Knowles et al., 1990a, and reprinted with permission.

creased liver damage (Billiar et al., 1990a). Further research will have to reconcile these observations with in vitro data suggesting that NO has a cytotoxic effect in hepatocytes (Curran et al., 1989). L-NMMA has been shown to potentiate LPS-induced gastrointestinal damage (Hutcheson et al., 1990) and the lethal effect of LPS in anesthetized rabbits (S. Moncada, R. M. J. Palmer, D. D. Rees, and C. E. Wright, unpublished observations). In contrast, L-NMMA reverses TNF-induced hypotension in dogs (Kilbourn et al., 1990) and inhibits LPS-induced hypotension in rats (Thiemermann and Vane, 1990).

It is likely, therefore, that the hypotension of endotoxemia or that induced by cytokines is mediated, at least in part, by the induction of the NO synthase in the vasculature. Furthermore, a low level of induction of the NO synthase may lead to vasodilation or hypotension, with or without tissue damage, depending on the rate of NO release. Interestingly, cirrhotic patients or animals with portocaval shunts show a hyperdynamic circulation consisting of vasodilation and increases in cardiac output (Murray et al., 1958; Schrier and Caramelo, 1988; Bosch et al., 1988). This has been attributed to elusive circulating vasoactive substances (Schrier and Caramelo, 1988). However, recent studies with L-NMMA in rats with experimental portal hypertension have shown attenuation of this hyperdynamic state (Pizcueta et al., 1991). Furthermore, it is also known that cirrhotic patients have increased levels of circulating endotoxin (Lumsden et al., 1988; Yomota et al., 1989) as well as an increased urinary excretion of cyclic GMP that is not associated with elevated levels of atrial natriuretic peptide (Miyase et al., 1990). In view of this it is likely that induction of NO synthesis in the peripheral circulation explains the hyperdynamic state of cirrhotic patients (Vallance and Moncada, 1991).

In summary, at this stage the consequences of induction of the NO synthase in a variety of tissues are not

yet fully understood. It is likely that in vivo, in addition to being cytotoxic, NO acts as a protective mechanism because of its powerful vasodilator action, an effect that cannot be observed in isolated cells or tissues. However, the interplay between these two properties of NO is not yet clearly understood and the net outcome of inhibiting NO generation is still uncertain. In view of these possibilities and apparent contradictory results the use of L-NMMA in humans for the treatment of septic shock should await the results of further research.

### G. Inhibition by Glucocorticoids of Immunologically Induced Formation of Nitric Oxide

Glucocorticoids, which are beneficial in the prevention or treatment of endotoxin shock in animals and humans (Hinshaw et al., 1980; Greisman, 1982; Nicholson, 1982; Hoffman et al., 1984), inhibit the induction of several proteins (for review, see Haynes and Murad, 1985). This led to the study of these compounds as inhibitors of the induction of the NO synthase.

The glucocorticoids, dexamethasone, hydrocortisone, and cortisol, inhibit the induction but not the activity of the $Ca^{2+}$-independent NO synthase in vitro in vascular endothelial cells (Radomski et al., 1990c), fresh vascular tissue (Rees et al., 1990a), the macrophage cell line J774 (Di Rosa et al., 1990), and the EMT-6 adenocarcinoma cells (O'Connor and Moncada 1991) after stimulation with LPS, either alone or in combination with IFN-γ. In addition, the in vitro inhibition of induction of the NO synthase by glucocorticoids was prevented by cortexolone (Radomski et al., 1990c; Di Rosa et al., 1990), a partial agonist on glucocorticoid receptors, indicating that this action is specific and therefore related to the pharmacological and probably some of the physiological effects of glucocorticoids. The induction by interleukin-1 and TNF of NO synthase in mesangial cells in vitro is also inhibited by dexamethasone (Pfeilschifter and Schwarzenbach, 1990). In vivo induction of NO synthase in liver, lung, and vascular tissue of rats after treatment with LPS was prevented by dexamethasone (Knowles et al., 1990c; fig. 15) and cortisol. This action of glucocorticoids, which occurs at the low concentrations achieved in plasma during the therapeutic use of these compounds, correlates with their anti-inflammatory potency and is not shared by progesterone.

The induction by LPS of the NO synthase in vascular tissue in vitro and the accompanying vascular relaxation, the hyporesponsiveness to vasoconstrictors, and the increase in cyclic GMP were inhibited by incubation with dexamethasone (Rees et al., 1990a; fig. 16). This suggests that immunologically induced release of NO may indeed explain at least some of the pathophysiological features of endotoxin shock.

### IV. Conclusions

The discovery of the formation of NO from L-arginine by mammalian tissues and the elucidation of some of its
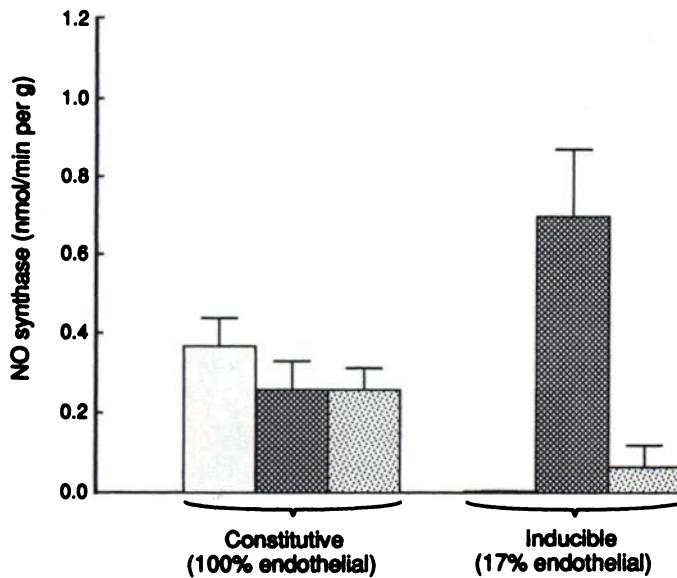
FIG. 15. The effect of dexamethasone on the constitutive and inducible NO synthases in the rat aorta. Rats were treated (i.p.) with vehicle (□), LPS (4 mg/kg; ▨) or LPS plus dexamethasone (3 mg/kg; ■) 6 h before preparation of cytosol from the thoracic aorta and spectrophotometric assay of NO synthase. The distribution of the NO synthases between the endothelial and the smooth muscle layer was determined by comparison with cytosols from aortae from which the endothelium had been removed by gentle rubbing. In tissues from untreated animals, constitutive NO synthase activity was entirely localized in the endothelial layer and this was not significantly different in preparations from animals treated with LPS or LPS plus dexamethasone. When assayed in the presence of ethyleneglycol bis($\beta$-aminoethyl ether)-N,N,N',N'-tetraacetic acid (1 mM) this constitutive activity was abolished in cytosols from untreated animals, but those from animals treated with endotoxin contained an NO synthase activity, the presence of which was attenuated by treatment with dexamethasone. Data from Knowles et al., 1990c.

biological roles has, in the last 4 years, thrown new light onto many areas of research. NO is released under physiological conditions by a constitutive, $Ca^{2+}$-dependent enzyme in response to receptor stimulation. This L-arginine:NO pathway is the transduction mechanism for the soluble guanylate cyclase and, as such, is among the mechanisms whereby cells regulate their own function or communicate with others.

In the cardiovascular system the release of NO acts as a general adaptive mechanism whereby the vascular endothelium responds to changes in its environment and regulates blood flow and blood pressure through an action on the vascular smooth muscle. In addition, NO regulates the interaction between the endothelium and the platelets and probably blood-borne cells, and it may also play a role in the control of vascular smooth muscle proliferation.

The generation of NO also acts as an autocrine regulatory system, for platelets do not seem to transfer NO to other platelets or cells but modulate their own ability to aggregate by generating NO. At present there are no other examples of this autocrine function. However, the increases in cyclic GMP that follow receptor stimulation
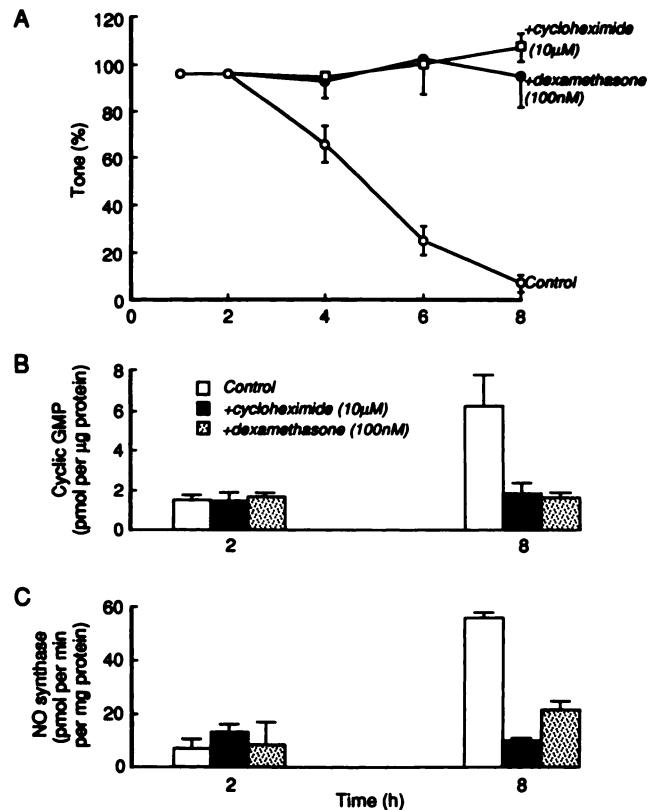


FIG. 16. The effect of cycloheximide and dexamethasone on the induction in endothelium intact aortic rings of a $Ca^{2+}$-independent NO synthase and its functional consequences. A, Time-dependent loss of tone of phenylephrine-contracted rings of rat aorta (control) was inhibited by cycloheximide (10 $\mu$M) and by dexamethasone (100 nM). B, Cyclic GMP levels (□) in rings of rat aorta at 2 h were not significantly affected by cycloheximide (■) or by dexamethasone (▨). However, after 8 h incubation in Krebs' buffer the basal cyclic GMP levels were significantly elevated and this elevation was prevented by both compounds. C, At 2 h there was no significant $Ca^{2+}$-independent NO synthase activity in the cytosol from rings of rat aorta from control animals (□) or from those treated with cycloheximide (■) or dexamethasone (▨). At 8 h, however, there was significant $Ca^{2+}$-independent NO synthase activity and this was inhibited by both compounds. Data from Rees et al., 1990a.

in some cells are likely to depend on NO generated by those same cells.

The importance of the L-arginine:NO pathway in the nervous system has yet to be established fully, although it is already known to be linked to the stimulation by the excitatory amino acids of specific receptors in the central nervous system. Because both the NO synthase and the soluble guanylate cyclase are widely and not uniformly distributed in the brain, it is likely that this pathway is associated with other mediator systems, not only in the central nervous system but also in both the sensory and motor areas of the peripheral nervous system. Furthermore, the presence of the NO synthase has been demonstrated in the adrenal medulla and the retina, where it may be involved in regulation of catecholamine release and in the gating mechanism for light-sensitive neurons, respectively.

The NO released by the constitutive enzyme may also play regulatory roles in other cells. So far, this enzyme has been found in the adrenal cortex and some epithelial cell lines, suggesting that it may participate in the regulation of the secretion or action of other hormones.

NO is also released after immunological stimulation by an enzyme that is inducible and $Ca^{2+}$ independent. This enzyme, which was originally described in the macrophage, releases NO as part of the host defence mechanism, because it has been shown to be cytotoxic or cytostatic for tumour cells and invasive organisms. More recently, the induction of this enzyme has been demonstrated in other cells and tissues not belonging to the reticuloendothelial system, suggesting that the release of NO might have other biological consequences, including pathological vasodilation and tissue damage.

The finding that this enzyme can be induced in vascular tissue in vitro by LPS, which may be present in physiological solutions or biological materials, is of special relevance to physiologists and pharmacologists, because the NO released will change the behaviour of their experimental models. In the past this release of NO, in vitro and probably in vivo, must have represented an unknown, confusing factor in otherwise well-planned and -executed experiments.

Resistance to infection and cancer can be enhanced in a nonspecific way by bacterial products, a fact known for more than a century since Fehleisen (1882) and later Coley (1893) used live streptococci to induce erysipelas in the treatment of patients with cancer. The biological basis of this phenomenon has been related to macrophage function (Old et al., 1961; Alexander and Evans, 1971; Hibbs et al., 1972). Present evidence indicates that this nonspecific immunity is related to the induction of an NO synthase. If this is the case, NO-dependent nonspecific immunity is a general phenomenon involving not only the reticuloendothelial system but also other cells, notably the vascular endothelium which is the most widespread of all tissues. Indeed, it is possible that the killing of *Rickettsia* by vascular endothelial cells activated with leukocyte culture supernatant (Wisseman and Waddell, 1983) is mediated through the release of NO via this synthase. In this context, the role of the lung and liver in NO-dependent nonspecific immunity is, therefore, crucial, because both organs are strategically placed in the circulation to serve as immunological filters.

We can conclude, therefore, that nonspecific immunity can be considered as nonspecific not only in terms of the stimuli that lead to its enhancement but also in terms of the variety of cells able to express it.

Immunologically generated NO, in addition to being cytostatic or cytotoxic for invading microorganisms or cancer cells, may also have similar adverse effects on host cells induced to express the NO synthase or in cells adjacent to these. Indeed, macrophages, hepatocytes, and

EMT-6 adenocarcinoma cells in which this pathway has been induced show signs of NO-dependent toxicity (Billiar et al., 1989; Albina et al., 1989b; Lepoivre et al., 1989; O'Connor and Moncada, 1991). The biological consequences of these changes, as well as the circumstances in which the release of NO leads to cell dysfunction or to cell death, await elucidation. However, some forms of local or systemic tissue damage associated with immunological conditions could prove to be related to the release of NO.

In addition to its effects on cell viability and thus proliferation, NO may also play a role in the normal regulation of the response of cells to mitogens. Changes in cyclic GMP have been associated with both the initiation and the control of cell proliferation in many cells (Seifert and Rudland, 1974; Weinstein et al., 1974). In view of the fact that NO can be released by the constitutive and by the inducible enzyme, it is important to differentiate between actions mediated via the cyclic GMP system and those resulting from NO acting as a cytotoxic/cytostatic agent.

Whether NO formed by the inducible enzyme also contributes to the cytotoxic actions of other cells that play a role in specific immunity is not yet known; however, induction of NO synthesis has been demonstrated in T-lymphocytes (Kirk et al., 1990).

At present the only NO synthase described in the brain is $Ca^{2+}$ dependent and constitutive, and animals treated with LPS do not show induction of a $Ca^{2+}$-independent enzyme in this tissue. The possibility that LPS did not penetrate the blood-brain barrier in these experiments and that glial cells, which are from the macrophage lineage, could express an inducible NO synthase cannot, however, be ruled out. If this is the case, then NO may be involved in some of the pathological changes observed in the brain in sepsis (Trump et al., 1975; Mela et al., 1979).

The finding that glucocorticoids inhibit the induction of this NO synthase is of special significance since it may explain, at least in part, the therapeutic and toxic action of these compounds and may reveal the potential importance of NO in a variety of conditions. Indeed, inhibition of the induction of the NO synthase may explain the antierythema and antiedema actions as well as the beneficial effect that glucocorticoids have on endotoxin shock, asthma, and rheumatoid arthritis, where NO may be responsible for pathological vasodilation and tissue damage. Furthermore, inhibition of the formation of NO may help to explain why glucocorticoids facilitate the spread of infections (Schaffner and Schaffner, 1987) and malignancy (Schultz et al., 1985; Hogan and Vogel, 1988) and prevent the consequences of delayed hypersensitivity in conditions such as transplant rejection (for review, see Haynes and Murad, 1985) or vasculitis (Conn, 1989).

The way in which L-arginine is made available to the NO pathway and the relative importance of this meta-

bolic route in relation to others for L-arginine metabolism remains to be elucidated. It seems that in vivo and in vitro, unless cells are maintained in L-arginine-free media, there is sufficient intracellular L-arginine to supply the $Ca^{2+}$-dependent NO synthase. However, when cells are stimulated in vitro to generate NO, it is then possible to supply exogenous L-arginine to this enzyme. Indeed, this seems to be the case in vascular endothelial cells, platelets, and neutrophils. Endothelial cells that have been depleted of L-arginine are able to synthesize this amino acid from endogenous sources (Hecker et al., 1990; Sessa et al., 1990), suggesting that under normal circumstances the availability of L-arginine is well regulated. A different situation may operate for the inducible, $Ca^{2+}$-independent enzyme because, once induced, this enzyme releases NO for long periods, and in macrophages in vitro, for example, it leads to a depletion of L-arginine in the medium or to their own death. A decrease in the levels of L-arginine, either locally or systemically, may be involved in hypertension, vasospasm, or atherosclerosis.

Furthermore administration of L-arginine may lead to a reduction in blood pressure or to an antithrombotic state. Moreover, it is likely that immunological reactions are accompanied by an increased utilization of L-arginine by the inducible enzyme, leading to its depletion either locally or systemically. Administration of arginine under these circumstances may result in an enhanced "nonspecific immunity" in terms of cytotoxicity, vasodilation, and other, as yet undefined, effects.

Although at present the precise mechanism for the biosynthesis of NO is unknown, three mechanisms have been proposed. One involves the deimination of arginine to form ammonia which is then oxidized to NO (Hibbs et al., 1987a); another suggests the formation of $N^G$-hydroxy-arginine via an NADPH- and tetrahydrobiopterin-dependent monoxygenation (Marletta et al., 1988); the third proposes that the $N^G$-hydroxy-arginine gives rise to hydroxylamine which is converted to NO by the action of catalase (DeMaster et al., 1989). All three mechanisms predict that the oxygen in NO would be derived from molecular oxygen and the ureido oxygen in citrulline would be derived from water. However, the recent studies using $^{18}O_2$ and mass spectrometry showing that both the constitutive and the inducible enzyme can be classified as $N^G,C^G$-dioxygenases indicate that these three proposed mechanisms are incorrect. Further work is now required to elucidate the reaction mechanism of these enzymes. These studies will also clarify whether NO is directly formed from L-arginine or whether there is a free radical intermediate involved in its formation (Arroyo et al., 1990).

Most of the details about the function, distribution, and interaction of these two mechanisms for the synthesis of NO in human physiology and pathology have yet to be studied. So far, information concerning the gener-

ation of NO in humans comes from platelets, neutrophils, and blood vessels and indirectly from studies that showed that prolonged exercise doubles urinary $NO_3^-$ in humans (Leaf et al., 1990a). L-Arginine: NO-mediated killing of *Mycobacterium* by human macrophages has recently been demonstrated (Denis, 1991).

Caution should also be exercised when interpreting results obtained with exogenous L-arginine, because this compound may have other actions not related to the L-arginine:NO pathway. Indeed, at present it is not yet clear whether the potent secretagogue effect of L-arginine in the pancreas and the pituitary gland or its effects on atherosclerosis, infection, and tumours (Barbul, 1986) are related to the synthesis of NO.

Finally, the implications of the synthesis of NO in terms of potential novel treatments for different diseases needs to be analysed and developed. It can, however, be predicted that, as with other fundamental biological discoveries, this will find its application in novel therapies.

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