Sequential induction of nitric oxide synthase by *Corynebacterium parvum* in different organs of the mouse

D.D. Rees, F.Q. Cunha, J. Assreuy, A.G. Herman & S. Moncada

Wellcome Research Laboratories, Langley Court, Beckenham, Kent BR3 3BS

- 1 The ability of *Corynebacterium parvum* (*C. parvum*) to induce nitric oxide (NO) synthase in the macrophage, spleen, liver, aorta, heart and brain, and to elevate plasma $\text{NO}_2^-/\text{NO}_3^-$ in the mouse was investigated. In addition, the relationship between NO synthase activity and blood pressure was studied.
- 2 *C. parvum* (100 mg kg^{-1} , i.p.) induced a time-dependent expression of a Ca^{2+} -independent NO synthase in the macrophage, spleen, liver, aorta and heart. The time course of induction of the NO synthase varied such that the maximum enzyme activity was at day 8 in the macrophage and liver, day 12 in the spleen and heart and day 16 in the aorta.
- 3 There was no significant induction of a Ca^{2+} -independent NO synthase in the brain, nor was there any change in the Ca^{2+} -dependent enzyme in this organ, during the study period.
- 4 *C. parvum* produced a gradual decrease in blood pressure, with a maximum fall at day 16 (from $108 \pm 1 \text{ mmHg}$ to $79 \pm 3 \text{ mmHg}$), which recovered gradually by day 28.
- 5 Plasma $\text{NO}_2^-/\text{NO}_3^-$ was significantly elevated between days 8 and 24, with a maximum increase at day 12.
- 6 These results show that *C. parvum* induces a Ca^{2+} -independent NO synthase in a number of tissues and that this induction occurs initially in macrophages and the liver. This suggests that induction of the NO synthase in the other tissues is secondary and probably the result of activation of macrophages and some cells of the liver.
- 7 Furthermore, the decrease in blood pressure induced by *C. parvum* is associated with the induction of NO synthase in the vasculature, whereas the increased concentration of plasma $\text{NO}_2^-/\text{NO}_3^-$ seems to result from the generation of NO by a number of tissues.

Keywords: *C. parvum*; plasma nitrite/nitrate; blood pressure; inducible nitric oxide synthase

Introduction

The L-arginine: nitric oxide (NO) pathway (Moncada *et al.*, 1991) is now known to be involved in a variety of physiological processes in the cardiovascular and nervous system where NO is generated via constitutive Ca^{2+} -dependent NO synthases (Moncada *et al.*, 1991; Snyder & Brecht, 1992). On the other hand, an inducible Ca^{2+} -independent NO synthase is expressed in phagocytic and other cells following activation by endotoxin (lipopolysaccharide; LPS) and/or cytokines. Much larger amounts of NO are generated by this enzyme, which is responsible for the cytotoxicity of macrophages towards certain micro-organisms and tumour cells and may account for the phenomenon of non-specific immunity (Moncada *et al.*, 1991). Endotoxin and/or cytokines also induce the expression of a Ca^{2+} -independent NO synthase in vascular endothelial and smooth muscle cells and cardiac myocytes. This accounts for the hypotension and probably the cardiac dysfunction characteristic of septic shock (Rees *et al.*, 1990a; Busse & Mulsch, 1990; Fleming *et al.*, 1991; Moncada, 1992; Wright *et al.*, 1992; Schulz *et al.*, 1992).

Administration of endotoxin *in vivo* activates macrophages, leading to the production of a variety of cytokines, including tumour necrosis factor (TNF) (Suffredini *et al.*, 1989; De Groote *et al.*, 1992; Oliver *et al.*, 1993), the maximal release of which occurs 2 h after endotoxin administration (Oliver *et al.*, 1993). These cytokines, in turn, induce the Ca^{2+} -independent NO synthase simultaneously in a variety of tissues, with maximal induction occurring after a further 4–12 h (Knowles *et al.*, 1990; Salter *et al.*, 1991; Cunha *et al.*, 1994).

Administration of the heat-inactivated gram-positive bacterium *Corynebacterium parvum* (*C. parvum*) has been shown to induce NO synthase in rat and mouse liver hepatocytes

in vivo, with a time course of several days (Geller *et al.*, 1993). *C. parvum* also induces the release of a number of cytokines over a period of weeks (Kawada *et al.*, 1990). Since the synthesis of NO is regulated by different cytokines in different cells, we wondered whether induction of NO synthase following *C. parvum* may vary significantly from one tissue to another. In view of this, we have investigated the effect of *C. parvum* on the induction of the NO synthase in various tissues of the mouse, and on the plasma concentrations of nitrite and nitrate ($\text{NO}_2^-/\text{NO}_3^-$), the breakdown products of NO. Furthermore, we have investigated the relationship between induction of the NO synthase and the blood pressure of the mouse.

Methods

Female CBA mice (Charles River, U.K. 30–35 g) were housed in a temperature-controlled room with water and food *ad libitum*. NO synthase activity in the spleen, liver, aorta, heart and brain, as well as blood pressure and plasma concentrations of $\text{NO}_2^-/\text{NO}_3^-$, were determined 2–28 days following administration of *C. parvum* (100 mg kg^{-1} , i.p., dissolved in sterile water, $\sim 0.3 \text{ ml}$ per mouse). In a separate series of experiments, the induction of NO synthase in peritoneal macrophages following *C. parvum* administration was also studied.

Measurement of plasma $\text{NO}_2^-/\text{NO}_3^-$

The animals were anaesthetized with isoflurane (2%) and blood samples ($\sim 0.5 \text{ ml}$) were obtained following exsanguination via the carotid artery. The plasma NO_2^- concentration was determined by first reducing the NO_3^- enzymatically, using NO_3^- reductase from *Aspergillus* species.

¹ Author for correspondence.

Briefly, plasma samples were diluted 1:4 or 1:10 with Milli-Q distilled water and incubated with assay buffer (composition, mM: KH_2PO_4 50, NADPH 0.6, FAD 5 and NO_3^- reductase 20 μM , pH 7.5) for 1 h at 37°C. A standard curve for NO_3^- was constructed by incubation of NaNO_3^- (1–100 μM) with the assay buffer. The resultant NO_2^- concentrations were determined by chemiluminescence as described previously (Palmer *et al.*, 1987) and expressed as the amount of total plasma nitrite and nitrate (NOx) in μM .

Measurement of NO synthase activity in tissues

Immediately after collection of the blood sample, the tissues were removed and washed briefly in Krebs' buffer (containing in mM: NaCl 118, KCl 4.8, CaCl_2 2.5, MgSO_4 1.2, KH_2PO_4 1.2, NaHCO_3 24 and glucose 11), gassed with 95% O_2 /5% CO_2 . The tissues were then freeze-clamped in liquid nitrogen and stored at -80°C .

On the day of assay, the frozen tissues were homogenized in a cooled stainless steel pestle and mortar and then placed in homogenizing buffer (pH 7.4; Tris 50 mM, sucrose 3.2 mM, dithiothreitol 1 mM, leupeptin 10 $\mu\text{g ml}^{-1}$, soybean trypsin inhibitor 10 $\mu\text{g ml}^{-1}$, aprotinin 2 $\mu\text{g ml}^{-1}$) and the tissue sonicated for 3 s ($\times 3$) at 4°C. The homogenate was centrifuged at 10,000 g for 20 min at 4°C. The resultant supernatant, containing both the soluble and particulate NO synthase was added to assay buffer (pH 7.2; containing KH_2PO_4^- 50 mM, MgCl_2 1 mM, CaCl_2 0.2 mM, valine 50 mM, L-citrulline 20 μM , L-arginine 20 μM , dithiothreitol 1 mM, NADPH 100 μM , tetrahydrobiopterin 3 μM , flavin adenine dinucleotide (FAD) 3 μM , flavin mononucleotide (FMN) 3 μM and L-[U- ^{14}C]-arginine 0.05 μCi ($\sim 1 \mu\text{M}$)). Following a 20 min incubation at 37°C, the reaction was terminated by removal of substrate by adding 1:1 (v/v) Milli-Q water/Dowex-AG50W (200–400, 8% cross linked, Na^+ -form). The resin was left to settle for 30 min at room temperature and the supernatant carefully removed. NO synthase activity in the supernatant was determined from the conversion of L-[U- ^{14}C]-arginine to [U- ^{14}C]-citrulline. The activity of the Ca^{2+} -dependent enzyme was determined as the difference between the [U- ^{14}C]-citrulline generated from control samples and samples containing EGTA (3 mM); the activity of the Ca^{2+} -independent enzyme was determined from the difference between samples containing EGTA (3 mM) and samples containing the NO synthase inhibitor, N^{ω} -iminoethyl-L-ornithine (L-NIO, 1 mM; Rees *et al.*, 1990b). The soluble protein content of the supernatant was determined by the Coomassie blue binding method using BIO-RAD protein reagent with bovine serum albumin as a standard. NO synthase activity was expressed as pmol NO $\text{min}^{-1} \text{mg}^{-1}$ protein. Due to the high arginase content and rapid utilisation of L-arginine in the liver, NO synthase levels in this tissue were determined from the conversion of L-[2,3,4,5 ^3H]-arginine to L[2,3,4,5 ^3H]-citrulline at 37°C for 10 min. Under these conditions the conversion of L-[^{14}C or ^3H]-arginine to L-[^{14}C or ^3H]-citrulline is linear in both assays.

Measurement of NO synthase in macrophages

The mice ($n = 16$) were killed by cervical dislocation. Culture medium (RPMI; 10 ml, 10 u ml^{-1} heparin) was injected into the peritoneum, agitated and withdrawn. The peritoneal suspension was centrifuged (10,000 g) for 10 min. The pellet was resuspended in distilled water for 5 s to lyse red blood cells, followed by phosphate buffered saline (PBS) and RPMI. The suspension was again centrifuged (10,000 g) for 10 min and the resulting pellet resuspended in RPMI. The macrophages were left to adhere in 12-well plates for 2 h. Non-adherent cells were discarded and adherent cells washed twice with fresh PBS and removed with a plastic scraper into homogenisation buffer. NO synthase activity was determined by the method described above. Macrophages from 4 mice were pooled to obtain each value.

Blood pressure

Mice ($n = 6$ per group) were anaesthetized with isoflurane (2%) at 0, 4, 8, 12, 16, 20, 24 and 28 days after *C. parvum* administration. A cannula line was implanted in the femoral artery, tunnelled subcutaneously to exit at the top of the back and connected to a swivel tether system for continuous monitoring of blood pressure. Normal physiological saline (154 mM) containing heparin (10 u ml^{-1}) was administered as a continuous infusion via the femoral artery (50 $\mu\text{l h}^{-1}$) to maintain patency of the blood pressure cannula line. Following a 1 h recovery from surgery, blood pressure was measured over the following 2 h period.

Materials

Corynebacterium parvum (*C. parvum*; Coparvax), N^{ω} -iminoethyl-L-ornithine (L-NIO; Wellcome Research Laboratories) and NO_3^- reductase (Boehringer, England) were obtained from the sources indicated. All other materials were obtained from Sigma (England).

Statistics

Statistical significance ($P < 0.05$) was analysed by a one-way ANOVA test for multiple comparisons followed by Dunnett-adjusted analysis. Results are expressed as means $\pm$ s.e.mean, $n = 3-10$.

Results

Induction of NO synthase in various tissues

Ca^{2+} -independent NO synthase Administration of *C. parvum* (100 mg kg^{-1} , i.p.) stimulated the expression of a Ca^{2+} -independent NO synthase with different time courses in the various tissues studied (Figure 1). Induction of this enzyme was maximal in harvested peritoneal macrophages and the liver at day 8 ($600 \pm 44 \text{ pmol min}^{-1} \text{mg}^{-1}$ protein and $1467 \pm 362 \text{ pmol min}^{-1} \text{mg}^{-1}$ protein respectively, $n = 4$ for each, $P < 0.05$), in the spleen and heart at day 12 ($16 \pm 0.8 \text{ pmol min}^{-1} \text{mg}^{-1}$ protein and $8 \pm 0.8 \text{ pmol min}^{-1} \text{mg}^{-1}$ protein respectively, $n = 3-10$ for each, $P < 0.05$) and in the aorta at day 16 ($124 \pm 12 \text{ pmol min}^{-1} \text{mg}^{-1}$ protein, $n = 3-8$, $P < 0.05$). *C. parvum* did not induce a Ca^{2+} -independent NO synthase in the brain.

Ca^{2+} -dependent NO synthase There was a smaller increase in activity of a Ca^{2+} -dependent NO synthase in the various tissues. This followed a similar time course to that of the Ca^{2+} -independent enzyme. The activity of the Ca^{2+} -dependent enzyme was maximal in harvested peritoneal macrophages and the liver at day 8 ($132 \pm 9 \text{ pmol min}^{-1} \text{mg}^{-1}$ protein; $n = 4$, $P < 0.05$, and $36 \pm 27 \text{ pmol min}^{-1} \text{mg}^{-1}$ protein respectively, $n = 3$), in the spleen and heart at day 12 ($2.4 \pm 0.4 \text{ pmol min}^{-1} \text{mg}^{-1}$ protein and $2.8 \pm 0.6 \text{ pmol min}^{-1} \text{mg}^{-1}$ protein respectively, $n = 3-10$ for each, $P < 0.05$) and in the aorta at day 16 ($54 \pm 18 \text{ pmol min}^{-1} \text{mg}^{-1}$ protein, $n = 3-8$, $P < 0.05$). There was no significant change in the activity of the Ca^{2+} -dependent NO synthase in the brain ($n = 4$; Figure 2).

Plasma $\text{NO}_2^-/\text{NO}_3^-$ measurements

The basal concentration of nitrite/nitrate (NOx) in the plasma was $7 \pm 3 \mu\text{M}$ ($n = 10$). *C. parvum* (100 mg kg^{-1} , i.p.) elevated plasma $\text{NO}_2^-/\text{NO}_3^-$ concentrations from day 4 ($13 \pm 2 \mu\text{M}$, $n = 5$, Figure 1), reaching a plateau at day 12 ($1006 \pm 23 \mu\text{M}$, $n = 5$, Figure 1, $P < 0.05$) and gradually returning to control values by day 28 ($22 \pm 11 \mu\text{M}$, $n = 5$, Figure 1).

Figure 1 Time course of the sequential induction of the Ca²⁺-independent NO synthase (NOS) in the liver (□), macrophages (■), spleen (△), heart (○) and aorta (●) and elevation of plasma concentrations of nitrite and nitrate (NO_x; *n* = 4, ▲, shaded area) following a single administration of *C. parvum* (100 mg kg⁻¹, i.p.; *n* = 3–10). Day 0 represents control, untreated group.

Figure 2 Ca²⁺-dependent NO synthase (NOS) activity in the brain following a single administration of *C. parvum* (100 mg kg⁻¹, i.p.; *n* = 4). There was no induction of the Ca²⁺-independent NO synthase over the time course studied. Day 0 represents control, untreated group.

Blood pressure measurements

Administration of *C. parvum* (100 mg kg⁻¹, i.p.) to the conscious mouse caused a progressive fall in mean arterial blood pressure (MABP) from a basal level of 108 ± 1 mmHg (*n* = 6, Figure 3) to a maximum fall at day 16 (79 ± 3 mmHg, *n* = 9, Figure 3, *P* < 0.01), which recovered gradually by day 28 (102 ± 1 mmHg, *n* = 4, Figure 3).

Discussion

Administration of endotoxin from gram-negative bacteria to animals induces the expression of a Ca²⁺-independent NO synthase which plays a role in the immunological defence mechanism against invading microorganisms and cancer cells and also mediates the excessive vasodilatation characteristic of septic shock (Moncada *et al.*, 1991). The induction of the NO synthase by endotoxin occurs in many tissues and organs (Knowles *et al.*, 1990; Salter *et al.*, 1991; Mitchell *et al.*, 1993; Cunha *et al.*, 1994) and is relatively short-lasting, with a time course which starts typically 2 h following endotoxin administration, peaks between 6–12 h and is largely over by 24 h (Knowles *et al.*, 1990; Salter *et al.*, 1991; Mitchell *et al.*, 1993;

Figure 3 Time course of induction of the Ca²⁺-independent NO synthase in the aorta (■, *n* = 3–8) and associated changes in mean arterial blood pressure (MABP; □, *n* = 4–10) following a single administration of *C. parvum* (100 mg kg⁻¹, i.p.). Day 0 represents control, untreated group. *P < 0.05, **P < 0.01.

Cunha *et al.*, 1994). This is accompanied by an increase in the concentration of NO₂⁻/NO₃⁻ in the plasma which follows a similar time course (Cunha *et al.*, 1994). Although not yet studied in detail, the gram-positive exotoxins appear to induce the expression of NO synthase in various tissues with a similar, short-lasting, time course of induction (Zembowicz & Vane, 1992; Cunha *et al.*, 1993).

There is another group of bacteria which, unlike those described above, induce the NO synthase after a long period of latency (1–2 days) and, in the organs studied, this induction lasts for a period of days or weeks. This group includes *Mycobacterium bovis* (strain bacillus Calmette Guérin; BCG, Stuehr & Marletta, 1987) and *C. parvum* (Billiar *et al.*, 1990; 1992). Both bacteria induce profound and long-lasting reticuloendothelial system stimulation, as shown by increased carbon clearance from the blood, associated with a persistence of the intact bacteria within the macrophage (Cummins, 1984). Interestingly, these have been used as immunostimulants and it is likely that this effect is mediated, in part, by NO. Many reports have shown that macrophages activated by endotoxin, *C. parvum* and/or cytokines mediate non-specific anti-microbial and anti-tumour activities (Tuttle & Cantrell, 1981) by a mechanism which involves induction of NO synthase (Hibbs *et al.*, 1990; Adams *et al.*, 1990; Moncada *et al.*, 1991).

We have now shown that *C. parvum* induces a sequential and differential induction of NO synthase, which is first expressed in the macrophage and the liver, followed by the spleen, heart and aorta. The fact that NO synthase induction occurs first in the macrophage and liver suggests that following the sequestration of the bacteria by macrophages and probably Kupffer cells in the liver, their activation and subsequent generation of cytokines stimulates the induction of NO synthase in other tissues. Induction in these tissues will depend on their responses to different cytokines. This leads to a temporal and differential expression of the enzyme in various tissues. Thus, our results show that 12–20 days after the administration of *C. parvum* there is induction of NO synthase in the vasculature which coincides with the development of a small but significant hypotension. The time course of changes in plasma NO₂⁻/NO₃⁻, on the other hand, appears to reflect the production of NO in several different organs, predominantly the liver and macrophage in which NO synthase activity is greatest.

Interestingly, relatively small increases in the Ca²⁺-dependent NO synthase were observed in all tissues over a similar time course. This may be artefactual in nature, since we

define Ca^{2+} -dependence as the residual activity after chelation of Ca^{2+} . Alternatively, induction of 'constitutive' NO synthases has been described previously in vascular tissue following shear stress (Nishida *et al.*, 1992) or oestrogen treatment (Weiner *et al.*, 1994). Whether induction of the Ca^{2+} -dependent enzyme occurs will therefore require further studies, including the measurement of mRNA for this enzyme.

The hypotension observed following administration of *C. parvum* was not as severe as that observed following treatment with endotoxin. The reasons for this are not clear at present, and detailed studies of the correlation between blood concentrations of $\text{NO}_2^-/\text{NO}_3^-$, induction of NO synthase in the vasculature and blood pressure are required. If, however, the maximum induction of NO synthase in the vessel wall is comparable after administration of endotoxin and *C. parvum* the question will remain as to why the fall in blood pressure following endotoxin is so severe and yet no hypotension has previously been described following *C. parvum* (Cummins, 1984). One possibility is that the cardiovascular system is able to adapt and compensate for the slow onset of the

induction of NO in the vasculature that occurs following *C. parvum* administration.

Whether immunostimulants other than *C. parvum* also induce this form of long-lasting sequential and differential induction of NO synthase in different tissues remains to be determined. Furthermore, it is worth investigating whether selective and long-lasting induction of the NO synthase in the reticuloendothelial system can be achieved. If so, it will be very important to assess the anti-infective and anti-cancer potential of such manipulation.

The authors thank Tara Andrews, Jayne Monkhouse and Angela Deakin for their assistance with this work and Annie Higgs for critical appraisal of the manuscript. F.Q.C. is on leave from the Dept. of Pharmacology, Faculty of Medicine, Universidade de Sao Paulo, Brazil and is supported by a fellowship from Fundacao de Amparo a Pesquisa do Estado de Sao Paulo. J.A. is on leave from the Dept. of Pharmacology, Universidade Federal do Rio de Janeiro, Brazil and is supported by a fellowship from Conselho Nacional de Desenvolvimento Cientifico e Tecnológico, Brazil. A.G.H. is on leave from the Dept. of Pharmacology, University of Antwerp, Belgium.

References

- ADAMS, L.B., HIBBS, J.B., TAINTOR, R.R. & KRAHENBUHL, J.L. (1990). Microbiostatic effect of murine macrophages for *Toxoplasma gondii*: role of synthesis of inorganic nitrogen oxides from L-arginine. *J. Immunol.*, **144**, 2725–2729.
- BILLIAR, T.R., CURRAN, R.D., HARBRECHT, B.G., STADLER, J., WILLIAMS, D.L., OCHOA, J.B., DI SILVIO, M., SIMMONS, R.L. & MURRAY, S.A. (1992). Association between synthesis and release of cGMP and nitric oxide biosynthesis by hepatocytes. *Am. J. Physiol.*, **262**, C1077–C1082.
- BILLIAR, T.R., CURRAN, R.D., STUEHR, D.J., STADLER, J., SIMMONS, R.L. & MURRAY, S.A. (1990). Inducible cytosolic enzyme activity for the production of nitrogen oxides from L-arginine in hepatocytes. *Biochem. Biophys. Res. Commun.*, **168**, 1034–1040.
- BUSSE, R. & MÜLSCH, A. (1990). Induction of nitric oxide synthase by cytokines in vascular smooth muscle. *FEBS Lett.*, **275**, 87–90.
- CUMMINS, C.S. (1984). *Corynebacterium parvum* and its fractions. *Immun. Ser.*, **25**, 163–190.
- CUNHA, F.Q., ASSREUY, J., MOSS, D.W., REES, D.D., LEAL, L.M.C., MONCADA, S. & CARRIER, M. (1994). Differential induction of nitric oxide synthase in various organs of the mouse during endotoxaemia: role of TNF α and IL-1 β . *Immunol.*, **81**, 211–215.
- CUNHA, F.Q., MOSS, D.W., LEAL, L.M.C., MONCADA, S. & LIEW, F.Y. (1993). Induction of macrophage parasitocidal activity by *Staphylococcus aureus* and exotoxins through the nitric oxide synthesis pathway. *Immunol.*, **78**, 563–567.
- DE GROOTE, D., ZANGERLE, P.F., GEVAERT, G., FASSOTTE, M.F., BEGUIN, Y., NOIZAT-PIRENNE, F., PIRENNE, J., GATHY, R., LOPEZ, M., DEHART, I., IGOT, D., BAUDRIHAYE, M., DELACROIX, D. & FRANCHIMONT, P. (1992). Direct stimulation of cytokines (IL-1 β , TNF α , IL-6, IL-2, IFN- γ and GM-CSF) in whole blood. I. Comparison with isolated PBMC stimulation. *Cytokine*, **4**, 239–248.
- FLEMING, I., GRAY, G.A., SCHOTT, C. & STOCLET, J.-C. (1991). Inducible but not constitutive production of nitric oxide by vascular smooth muscle cells. *Eur. J. Pharmacol.*, **200**, 375–376.
- GELLER, D.A., NUSSLER, A.K., SILVIO, M., LOWENSTEIN, C.J., SHAPIRO, R.A., WANG, S.C., SIMMONS, R.L. & BILLIAR, T. (1993). Cytokines, endotoxin, and glucocorticoids regulate the expression of inducible nitric oxide synthase in hepatocytes. *Proc. Natl. Acad. Sci. U.S.A.*, **90**, 522–526.
- HIBBS, J.B., TAINTOR, R.R., VAVRIN, Z., GRANGER, D.L., DRAPIER, J.-C., AMBER, I.J. & LANCASTER, J.R. (1990). Synthesis of nitric oxide from a terminal guanidino nitrogen atom of L-arginine: a molecular mechanism regulating cellular proliferation that targets intracellular iron. In *Nitric Oxide L-Arginine: a Bioregulatory System*. ed. Moncada, S. & Higgs, E.A. pp. 189–225. Amsterdam: Elsevier Science Publishers.
- KAWADA, N., MIZOGUCHI, Y., SHIN, T., TSUTSUI, H., KOBAYASHI, K., MORISAWA, S., MONNA, T. & YAMAMOTO, S. (1990). Synthesis of eicosanoids by *Propionibacterium acnes* – elicited liver adherent cells and their effect on production of interleukin 1 and tumor necrosis factor. *Prostaglandins Leukot. Essent. Fatty Acids*, **41**, 187–193.
- KNOWLES, R., MERRETT, M., SALTER, M. & MONCADA, S. (1990). Differential induction of brain, lung and liver nitric oxide synthase by endotoxin in the rat. *Biochem. J.*, **270**, 833–836.
- MITCHELL, J., KOHLHAAS, K.L., SORRENTINO, R., WARNER, T., MURAD, F. & VANE, J.R. (1993). Induction by endotoxin of nitric oxide synthase in the rat mesentery: lack of effect on action of vasoconstrictors. *Br. J. Pharmacol.*, **109**, 265–270.
- MONCADA, S. (1992). The L-arginine: nitric oxide pathway. *Acta Physiol. Scand.*, **145**, 201–227.
- MONCADA, S., PALMER, R.M.J. & HIGGS, E.A. (1991). Nitric oxide: physiology, pathophysiology and pharmacology. *Pharmacol. Rev.*, **43**, 109–142.
- NISHIDA, K., HARRISON, D.G., NAVAS, J.P., FISHER, A.A., DOCKERY, S.P., UEMATSU, M., NEREM, R.M., ALEXANDER, R.W. & MURPHY, T.J. (1992). Molecular cloning and characterization of the constitutive bovine aortic endothelial cell nitric oxide synthase. *J. Clin. Invest.*, **90**, 2092–2096.
- OLIVER, J.C., BLAND, L.A., OETTINGER, C.W., ARDUINO, M.J. & MCALLISTER, S.K. (1993). Cytokine kinetics in an in vitro whole blood model following an endotoxin challenge. *Lymphokine Cytokine Res.*, **12**, 115–120.
- PALMER, R.M.J., FERRIGE, A.G. & MONCADA, S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature*, **327**, 524–526.
- REES, D.D., CELLEK, S., PALMER, R.M.J. & MONCADA, S. (1990a). Dexamethasone prevents the induction by endotoxin of a nitric oxide synthase and the associated effects on vascular tone: an insight into endotoxin shock. *Biochem. Biophys. Res. Commun.*, **173**, 541–547.
- REES, D.D., PALMER, R.M.J., SCHULZ, R., HODSON, H.F. & MONCADA, S. (1990b). Characterisation of three inhibitors of endothelial nitric oxide synthase in vitro and in vivo. *Br. J. Pharmacol.*, **101**, 746–752.
- SALTER, M., KNOWLES, R.G. & MONCADA, S. (1991). Widespread tissue distribution, species distribution and changes in activity of Ca^{2+} -dependent and Ca^{2+} -independent nitric oxide synthases. *FEBS Lett.*, **291**, 145–149.
- SCHULZ, R., NAVA, E. & MONCADA, S. (1992). Induction and potential biological relevance of a Ca^{2+} -independent nitric oxide synthase in the myocardium. *Br. J. Pharmacol.*, **105**, 575–580.
- SNYDER, S.H. & BREDET, D.S. (1992). Biological roles of nitric oxide. *Scientific American*, May, 28–35.
- STUEHR, D.J. & MARLETTA, M.A. (1987). Induction of nitrite/nitrate synthesis in murine macrophages by BCG infection, lymphokines, or interferon- γ . *J. Immunol.*, **139**, 518–525.
- SUFFREDINI, A.F., FROMM, R.E., PARKER, M.M., BRENNER, M., KOVACS, J.A., WESLEY, R.A. & PARRILLO, J.E. (1989). The cardiovascular response of normal humans to the administration of endotoxin. *N. Engl. J. Med.*, **321**, 280–287.
- TUTTLE, R.L. & CANTRELL, J. (1981). *C. Parvum* – determinants of biological activity. In *Augmenting Agents in Cancer Therapy*. ed. Hersh, E.M., Chirigoz, M.A. & Mastrangelo, M.J. pp. 53–69. New York: Raven Press.

WEINER, C.P., LIZASOAIN, I., BAYLIS, S.A., KNOWLES, R.G., CHARLES, I.G. & MONCADA, S. (1994). Induction of calcium-dependent nitric oxide synthases by sex hormones. *Proc. Natl. Acad. Sci. U.S.A.*, **91**, 5212–5216.

WRIGHT, C.E., REES, D. & MONCADA, S. (1992). Protective and pathological roles of nitric oxide in endotoxin shock. *Cardiovasc. Res.*, **26**, 48–57.

ZEMBOWICZ, A. & VANE, J.R. (1992). Induction of nitric oxide synthase activity by toxic shock syndrome toxin 1 in a macrophage-monocyte cell line. *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 2051–2055.

(Received April 5, 1994
Revised September 1, 1994
Accepted October 4, 1994)