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Feedback inhibition of nitric oxide synthase activity by nitric oxide

J. Assreuy, F.Q. Cunha, *F.Y. Liew & [1]S. Moncada

The Wellcome Research Laboratories, Langley Court, Beckenham, Kent BR3 3BS and *Department of Immunology, University of Glasgow, Glasgow G11 6NT

1 A murine macrophage cell line, J774, expressed nitric oxide (NO) synthase activity in response to interferon-gamma (IFN-γ, 10 u ml^{-1}) plus lipopolysaccharide (LPS, 10 ng ml^{-1}). The enzyme activity was first detectable 6 h after incubation, peaked at 12 h and became undetectable after 48 h.

2 The decline in the NO synthase activity was not due to inhibition by stable substances secreted by the cells into the culture supernatant.

3 The decline in the NO synthase activity was significantly slowed down in cells cultured in a low L-arginine medium or with added haemoglobin, suggesting that NO may be involved in a feedback inhibitory mechanism.

4 The addition of NO generators, S-nitroso-acetyl-penicillamine (SNAP) or S-nitroso-glutathione (GSNO) markedly inhibited the NO synthase activity in a dose-dependent manner. The effect of NO on the enzyme was not due to the inhibition of *de novo* protein synthesis.

5 SNAP directly inhibited the inducible NO synthase extracted from activated J774 cells, as well as the constitutive NO synthase extracted from the rat brain.

6 The enzyme activity of J774 cells was not restored after the removal of SNAP by gel filtration, suggesting that NO inhibits NO synthase irreversibly.

Keywords: Nitric oxide; nitric oxide synthase inhibition; macrophage; J774 cells

Introduction

Nitric oxide (NO), derived from molecular oxygen and the guanidino nitrogen of L-arginine, is involved in a variety of biological functions (reviewed in Moncada *et al.*, 1991). Nitric oxide is the transduction mechanism for the soluble guanylate cyclase responsible for endothelium-dependent vascular relaxation, modulation of platelet aggregation and some forms of central and peripheral neurotransmission (reviewed in Moncada *et al.*, 1991). Nitric oxide also contributes to the cytotoxic and cytostatic actions of macrophages activated by various immunological stimuli (Hibbs *et al.*, 1988). The production of NO is catalysed by the enzyme NO synthase, of which there are at least two different types. One is constitutive and is Ca^{2+}/calmodulin-dependent. The other is inducible by cytokines and bacterial lipopolysaccharide (LPS) and is Ca^{2+}-independent. The activity of these enzymes can be specifically and stoichiometrically inhibited by structural analogues of L-arginine such as L-N^G-monomethyl arginine (L-NMMA).

The generation of NO by the inducible NO synthase is tightly regulated. Indeed, a number of cytokines such as transforming growth factor-beta (TGF-β) (Ding *et al.*, 1990), interleukin-4 (IL-4) (Liew *et al.*, 1991) and IL-10 (Cunha *et al.*, 1992) are effective in inhibiting the expression of the NO synthase. Furthermore, the induction of NO synthase in macrophages *in vitro* is accompanied by a steep increase in nitrite (NO_2^-) accumulation in the culture supernatant, which reaches a plateau 40–60 h after the initial stimulus (Stuehr & Marletta, 1987; Takema *et al.*, 1991). This suggests that there may also be a self-regulatory mechanism modulating the activity of NO synthase. We show here that NO can act as a negative feedback mechanism for its own synthesis.

[1] Author for correspondence.

Methods

Materials

The murine macrophage cell line J774 was obtained from American Tissue Culture Collection (ATCC). Cells were maintained in culture medium (DMEM [Gibco] supplemented with 10% foetal calf serum [FCS], 2 mM L-glutamine, 100 u ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 20 mM glucose). For this standard DMEM, the initial L-arginine concentration was 351 ± 10.2 μM ($n = 9$). In some experiments, DMEM with a low L-arginine concentration (13.5 ± 0.5 μM; $n = 3$) was used. The residual L-arginine was due to the amino acid present in FCS which was not dialysed. Murine recombinant IFN-γ was kindly provided by Dr G. Adolf, Ernst Boehringer-Institut fur Arzneimettel-Forschung, Vienna, Austria. LPS from *E. coli* (026:B6) was obtained from Difco. S-nitroso-acetyl-penicillamine (SNAP) was kindly provided by Dr H. Hodson (Department of Medicinal Chemistry, The Wellcome Research Laboratories, Beckenham). S-nitroso-glutathione (GSNO), synthesized according to the method of Hart (1985), was kindly provided by Dr A. Noronha-Dutra (Middlesex Hospital, University of London). 8-Bromo-guanosine 3′:5′-cyclic monophosphate (8-Br-cyclic GMP) was purchased from Sigma. L-[^{3}H]-leucine (specific activity 120 Ci mmol^{-1}; 4.44 TBq mmol^{-1}) and L-[2,3,4,5-^{3}H]-arginine (specific activity 62 Ci mmol^{-1}; 2.3 TBq mmol^{-1}) were obtained from the Radiochemical Centre, Amersham (UK). All other reagents were purchased from Sigma (Poole, England).

Induction of NO synthase activity and NO_2^- synthesis

J774 cells were cultured for 2 h at 37°C in an atmosphere of 5% CO_2 in 12-well Costar plates at 2 × 10^6 cells per 2 ml of DMEM. To each well, 2 ml of medium alone (control) or medium containing the stimulus (10 u ml^{-1} IFN-γ plus

10 ng ml^{-1} LPS) was added. The activity of NO synthase and the NO_2^- concentration were determined 6, 12, 24 and 48 h after activation.

Effect of cell supernatant on NO synthase levels

Cells were left in contact with or without the stimulus for 4 h, washed and cultured in fresh medium for another 12 h. Preliminary experiments demonstrated that the levels of NO synthase attained with this short period of exposure were the same as when the stimulus was left for longer periods. The supernatant was collected, filtered through a 0.2 μm filter and diluted 1:1 with fresh DMEM. Fresh J774 monolayers were pre-incubated with this culture supernatant (2 ml per well) for 12 h followed by stimulation with IFN-γ plus LPS. Nitric oxide synthase activity was determined 12 h after stimulation.

Effects of NO donors, 8-Br-cyclic GMP and haemoglobin on NO synthase levels

All experiments with SNAP, GSNO or 8-Br-cyclic GMP were carried out in low L-arginine medium to avoid interference by endogenously-produced NO. The NO donors and the cyclic nucleotide analogue were added together with the stimulus and the enzyme activity was assayed 12 h later. To test the effects of these compounds on the total protein synthesis, cells were pulsed with L-[^{3}H]-leucine (1 μCi per well; 2 h, 37°C) 4 h or 6 h after the stimulation. The medium was removed and the cells were washed with PBS containing 2 mM cold L-leucine and finally with 10% and 5% trichloracetic acid. The radioactivity in the monolayers was counted in a liquid scintillation counter (LKB, Sweden). The experiments with haemoglobin were carried out in standard DMEM.

Effect of SNAP on the inducible and constitutive NO synthases

Cytosol (20,000 *g*) from J774 cells was prepared by sonicating cells activated by 12 h incubation with the stimulus and used as the source of inducible enzyme. Rat brain cytosol was prepared by homogenizing brains in 0.1 M HEPES/1 mM dithiothreitol (DTT), pH 7.4, in a ratio of 1:5 (w/v) followed by centrifugation (20,000 *g*). The supernatant was used as the source of constitutive enzyme. Aliquots of both cytosols containing 5 μM tetrahydrobiopterin were incubated for 30 min at room temperature in the reaction mixture for the citrulline assay (see below) containing 0, 30, 100, 300 or 1000 μM of SNAP. The addition of SNAP or GSNO did not alter the pH of the medium. The citrulline assay was used because NO released by SNAP interferes with the spectrophotometric assay (see below).

To assess whether removal of SNAP would restore the enzyme activity, stimulated J774 cytosol was incubated with 1 mM of SNAP or buffer for 1 h at room temperature. These mixtures were then passed through a Sephadex G-50 (2.5 ml volume) column which was equilibrated and eluted in 0.1 M HEPES/1 mM DTT/1 mM EDTA/100 μM NADPH/5 μM tetrahydrobiopterin to avoid loss of enzyme activity by removal of cofactors. Fractions of 0.25 ml were collected. Peak enzyme activity appeared in fraction 6 while the SNAP appeared from fraction 10 onwards. The enzyme activity in the various fractions and in the controls was determined by the citrulline assay (see below).

Assays of NO synthase activities

Spectrophotometric assay The cell monolayers were washed with PBS and sonicated after addition of 0.25 ml of buffer (0.1 M HEPES/1 mM DTT/1 mM EDTA, pH 7.4). The cytosol (20,000 *g*) was assayed for NO synthase activity by the spectrophotometric assay based on the conversion of oxy- to methaemoglobin as described previously (Feelisch & Noack, 1987). The protein content of the cytosol was determined by the Coomassie Blue binding method (Pierce Chemical). Activity was expressed as pmol of NO mg^{-1} protein min^{-1}. The NO synthase activity in the positive controls varies between 600–1000 pmol NO mg^{-1} protein min^{-1} in different experiments. This probably reflects stages of differentiation of the cell line in different passages.

Citrulline assay This was performed by the conversion of radioactive L-arginine to L-citrulline as previously described (Salter *et al.*, 1991), except that it was carried out at room temperature for 30 min and [^{3}H]-L-arginine was used. Activity was expressed as pmol of citrulline mg^{-1} protein min^{-1}.

Measurement of NO_2^-

The concentration of NO_2^- in the supernatants was determined by chemiluminescence as previously described (Palmer *et al.*, 1987).

Statistical analysis

Results are expressed as mean ± s.e.mean and statistical significance ($P<0.05$) was analysed by Student's *t* test. In the figures, vertical bars = 1 s.e.mean; where no bar is seen, it is covered by the symbol.

Results

Time course of NO synthase activation and NO_2^- production

After 6 h of exposure to IFN-γ plus LPS a small amount of NO_2^- was detectable in the culture supernatant, increasing until 24 h and remaining essentially the same at 72 h (Figure 1). The NO synthase activity (measured by the spectrophotometric method) in the cytosolic fraction showed a high activity by 6 h, which peaked at 12 h, decreased thereafter to a low level by 48 h and was not detectable by 72 h (Figure 1). At this time 70–80% of the cells remained viable, as judged by trypan blue exclusion (data not shown).

There was no difference in the levels of enzyme activity in

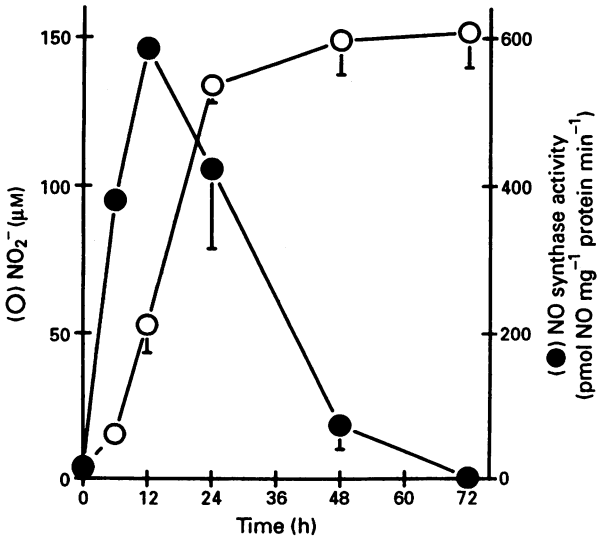

Figure 1 Time-course of NO_2^- accumulation (○) and NO synthase activity (●) in J774 cells stimulated with interferon gamma (IFN-γ, 10 u ml^{-1}) plus lipopolysaccharide (LPS, 10 ng ml^{-1}). Medium was standard DMEM. NO synthase activity was assayed by the spectrophotometric method. Vertical bars = 1 s.e.mean of triplicate wells. Results are representative of 3 experiments.

the cells which were pre-incubated with the supernatant derived from stimulated and non-stimulated cells before stimulation with IFN-γ plus LPS for 12 h (611 ± 73 vs 519 ± 19 pmol NO mg^{-1} protein min^{-1}, respectively, $n = 3$; $P > 0.05$).

Effects of haemoglobin or low L-arginine on NO synthase levels

Low concentrations of NO_2^- (10 ± 1.03 μM; $n = 3$) were found in the supernatant of cells stimulated in low L-arginine DMEM when compared to the NO_2^- concentrations in the supernatant from cells stimulated for 12 h in standard DMEM (137.1 ± 3.4 μM, $n = 3$). The presence of haemoglobin also reduced the NO_2^- concentrations in the supernatant (71.7 ± 1.6 mM; Figure 2a). In contrast, when the enzyme activity from those cells was studied by the spectrophotometric method, the peak enzyme activity in low L-arginine DMEM was markedly increased and the decay of this activity was slowed down when compared to that observed after activation in standard DMEM. The presence of haemoglobin also increased the peak activity of the enzyme by 50% and reduced the rate of its decay (Figure 2b).

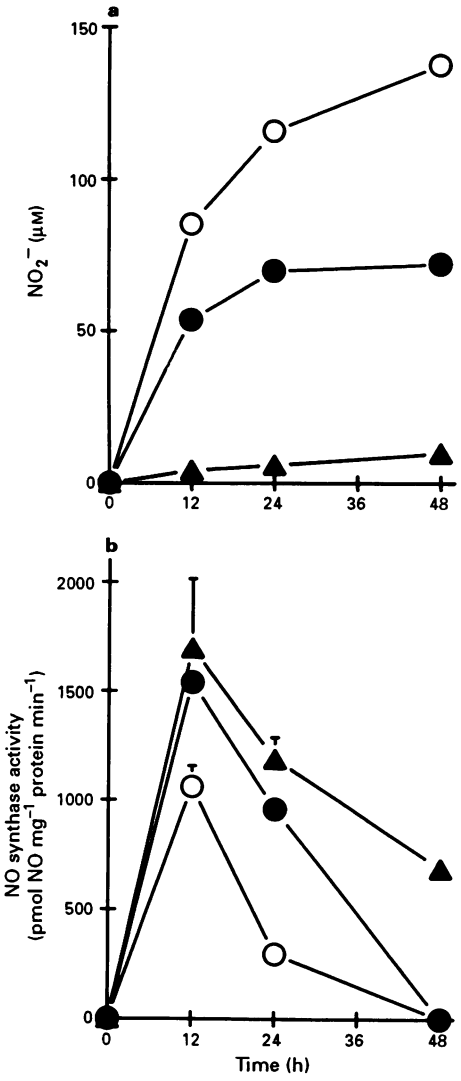

Figure 2 Time-course of NO_2^- accumulation (a) and NO synthase activity (b) of J774 cells activated with interferon gamma (IFN-γ, 10 u ml^{-1}) plus lipopolysaccharide (LPS, 10 ng ml^{-1}), in standard DMEM (○), in the presence of 200 μM of haemoglobin (●), or in low L-arginine DMEM (▲). NO synthase activity was assayed by the spectrophotometric method. Each point is the mean ± s.e.mean of triplicate wells. Results are representative of 2 experiments.

Effects of SNAP, GSNO and 8-Br-cyclic GMP

When added to the cells at the same time as the stimulus, the NO donors SNAP and GSNO inhibited the enzyme activity in a dose-dependent manner, whereas 8-Br-cyclic GMP was without effect (Figure 3). The inhibitory effect of SNAP and GSNO was due to NO, since neither acetyl-penicillamine nor glutathione (which do not release NO) had any effect on NO synthase activity (779 ± 144 and 734 ± 90 pmol NO mg^{-1} protein min^{-1}, respectively, $n = 3$). Neither SNAP nor 8-Br-cyclic GMP (10–100 μM) affected the macrophage total protein synthesis assayed 4, 8 or 12 h after stimulation ($n = 3$ for each concentration and each time).

Effect of SNAP on NO synthase activity in a cell-free system

When SNAP was incubated with the cytosolic fraction of stimulated J774 cells, it inhibited the NO synthase activity (measured by the citrulline assay) in a dose-dependent manner (Figure 4). In parallel experiments, the enzyme activity of the constitutive NO synthase extracted from rat brain was similarly inhibited by SNAP (Figure 4). The removal of SNAP by gel filtration did not restore the NO synthase activity (Figure 5).

Discussion

The NO synthase activity induced in macrophages following activation by immunological stimuli decays rapidly. Since supernatants of stimulated cells failed to affect the production of NO by other stimulated J774 cells, such decay is not likely to be due to inhibitory stable substances produced by the cells and present in the culture supernatant. We have now provided evidence that the decay in NO synthase activity is due, at least in part, to feedback inhibition by NO. Thus, low concentrations of L-arginine (which limit NO synthesis), or the presence of haemoglobin (which scavenges NO), increased the activity and slowed the decay of NO synthase activity. In contrast, the addition of SNAP and GSNO,

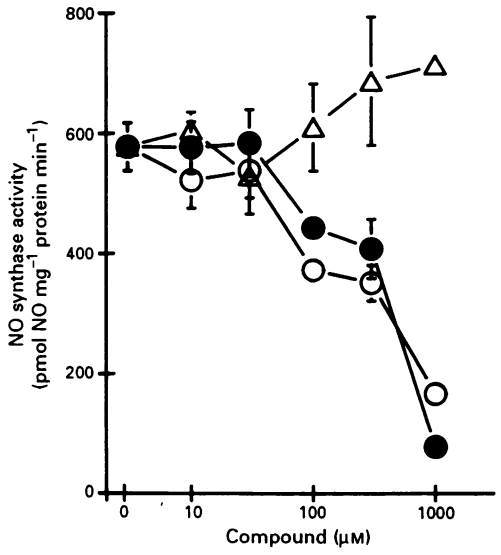

Figure 3 Effect of NO donors and 8-Br-cyclic GMP on the NO synthase activity in J774 cells. S-nitroso-acetyl-penicillamine (SNAP, ○), S-nitroso-glutathione (GSNO, ●) or 8-Br-cyclic GMP (△) were added together with the stimuli (10 u ml^{-1} interferon gamma plus 10 ng ml^{-1} lipopolysaccharide) and the enzyme activity assayed 18 h later by the spectrophotometric assay. Each point is the mean ± s.e.mean of triplicate wells. Results are representative of 2 experiments.

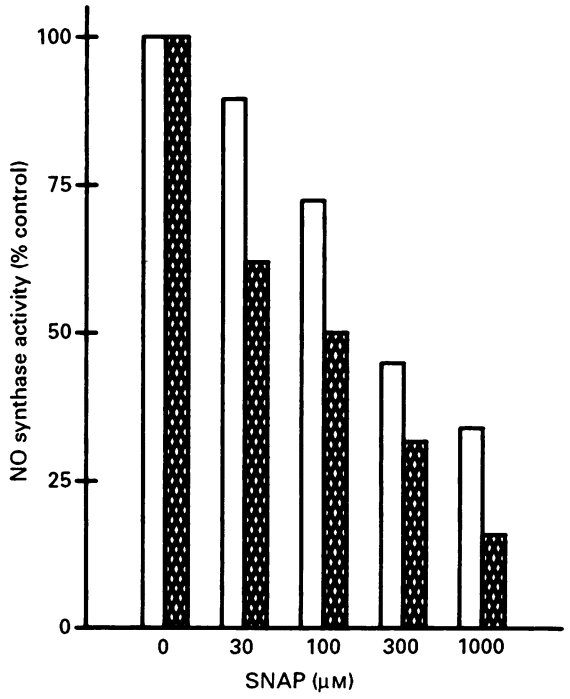

Figure 4 Inhibitory effect of S-nitroso-acetyl-penicillamine (SNAP) on NO synthase extracted from the cytosol of stimulated J774 cells (open columns) and from rat brain (cross-hatched columns). Aliquots of both cytosols were incubated with SNAP in the presence of 5 μM tetrahydrobiopterin. After 30 min at room temperature, the NO synthase activity was assayed by the citrulline assay. Original activities were 1000 and 300 pmol citrulline mg^{-1} protein min^{-1} for J774 and rat brain cytosol, respectively. Similar results were obtained with a 60 min incubation time. Data are representative of 2 experiments each with triplicate samples.

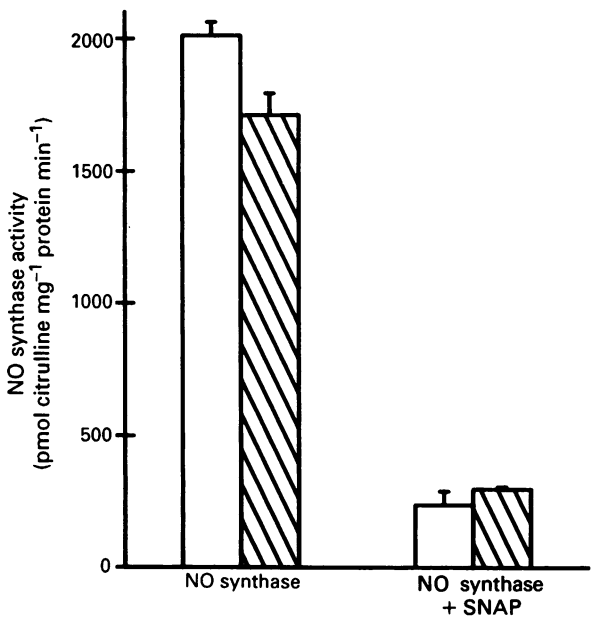

Figure 5 Gel-filtration analysis of S-nitroso-acetyl-penicillamine (SNAP)-inactivated NO synthase. Aliquots of cytosol extracted from activated J774 cells and containing 5 μM tetrahydrobiopterin were incubated with buffer or with 1 mM of SNAP for 60 min at room temperature. Part of the mixtures was then gel filtered through a Sephadex G-50 column. NO synthase activity was assayed by the citrulline assay. The data are total activity of non-fractionated enzyme (open columns) or of fractionated (pooled fractions 5–7) enzyme (hatched columns). Results represent mean ± s.e.mean of 3 experiments.

which produce NO, strongly inhibited the enzyme activity either in whole cells or in a cell-free system. The latter finding clearly indicates that NO inhibits the enzyme directly. The requirement of relatively high concentrations of NO donors in this system is probably due to the slow release of NO by these compounds. NO is released by GSNO at a rate of 0.02 nmol min^{-1} (Radomski *et al.*, 1992). Since the dose-response curves of SNAP and GSNO on the inhibition of NO synthase are very similar, it is likely that SNAP releases NO at a similar rate.

SNAP also inhibited the activity of the constitutive brain NO synthase. An inhibition of the particulate but not the soluble constitutive NO synthase in endothelial cells caused by superoxide has been suggested (Mitchell *et al.*, 1991). The mechanism by which NO inhibits NO synthase activity is at present unknown. The concentrations of NO released by SNAP, although sufficient to inhibit the enzyme activity, had no effect on the total protein synthesis of the macrophages. The inhibition did not appear to be mediated by cyclic GMP, since 8-Br-cyclic GMP had no effect. It is also unlikely that NO inhibits the NO synthase activity indirectly by affecting the levels of NADPH or tetrahydrobiopterin, co-factors essential for NO synthase activity, since excess concentrations of these compounds were added in the NO synthase assays. It may be that NO has a direct effect on the activity of NO synthase. This is consistent with our finding that SNAP could inhibit the enzyme activity in a cell-free system. Furthermore, the removal of SNAP from the incubate did not restore the enzyme activity. This suggests that the inhibition of NO synthase by NO may be irreversible. Whether other free radicals can also inhibit NO synthase is at present unknown.

Nitric oxide has been shown to induce S-nitrosylation of four cysteine residues of glyceraldehyde-3-phosphate dehydrogenase which are essential for the catalytic function, thereby inhibiting the enzyme activity (Molina y Vedio *et al.*, 1992). Mouse macrophage inducible enzyme has been shown to have 24 cysteine residues (Lyons *et al.*, 1992; Xie *et al.*, 1992), the potential sites of S-nitrosylation. Nitric oxide has also been shown to react with amino groups of proteins (Moriguchi *et al.*, 1992). It was recently shown that NO synthase is a haem protein (White & Marletta, 1992). It is possible that NO binds directly to the haem moiety of the enzyme. Whether NO inhibits the synthase activity by these or other mechanisms remains to be established. It is also unclear whether, in addition to its direct inhibitory effect on the enzyme, NO also inhibits the induction process leading to the expression of the NO synthase.

Earlier studies have demonstrated that a number of cytokines (TGF-β, IL-4 and IL-10) can inhibit the induction of NO synthase in macrophages. Our present data suggest an additional regulatory process of NO synthesis via a feedback mechanism by NO.

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References

CUNHA, F.Q., MONCADA, S. & LIEW, F.Y. (1992). Interleukin-10 (IL-10) inhibits the induction of nitric oxide synthase by interferon-γ in murine macrophages. *Biochem. Biophys. Res. Commun.*, **186**, 1155–1159.

DING, A., NATHAN, C.F., GRAYCAR, J., DERYNCK, R., STUEHR, D.J. & SRIMAL, S. (1990). Macrophage deactivating factor and transforming growth factors-β1, β2 and β3 inhibit induction of macrophage nitrogen oxide synthesis by IFN-γ. *J. Immunol.*, **145**, 940–944.

FEELISCH, M. & NOACK, E.A. (1987). Correlation between nitric oxide formation during degradation of organic nitrates and activation of guanylate cyclase. *Eur. J. Pharmacol.*, **139**, 19–30.

HART, T.W. (1985). Some observations concerning the S-nitroso and S-phenylsulphonyl derivatives of L-cysteine and glutathione. *Tetrahedron Lett.*, **26**, 2013–2016.

HIBBS, J.B.Jr., TAINTOR, R.R., VAVRIN, Z. & RACHLIN, E.M. (1988). Nitric oxide: a cytotoxic activated macrophage effector molecule. *Biochem. Biophys. Res. Commun.*, **157**, 87–94.

LIEW, F.Y., LI, Y., SEVERN, A., MILLOTT, S., SCHMIDT, J., SALTER, M. & MONCADA, S. (1991). A possible novel pathway of regulation by murine T helper type-2 (Th_2) cells of a T_h1 cell activity via the modulation of the induction of nitric oxide synthase on macrophages. *Eur. J. Immunol.*, **21**, 2489–2494.

LYONS, C.R., ORLOFF, G.J. & CUNNINGHAM, J.M. (1992). Molecular cloning and functional expression of an inducible nitric oxide from a murine macrophage cell line. *J. Biol. Chem.*, **287**, 6370–6374.

MITCHELL, J.A., SHENG, H., WARNER, T.D., POLLOCK, J.S., FORSTERMANN, U. & MURAD, F. (1991). Relationship between endothelial cell activation and subsequent NO synthase activity: inhibition by oxygen derived free radicals. *Br. J. Pharmacol.*, **104**, 117P.

MOLINA Y VEDIO, L., McDONALD, B., BRUNE, B. & LAPETINA, E.G. (1992). Nitric oxide-induced S-nitrosylation of glyceraldehyde-3-phosphate dehydrogenase inhibits enzymatic activity and increases endogenous ADP-ribosylation. *J. Biol. Chem.*, (in press).

MONCADA, S., PALMER, R.M.J. & HIGGS, E.A. (1991). Nitric oxide: physiology, pathophysiology and pharmacology. *Pharmacol. Rev.*, **43**, 109–142.

MORIGUCHI, M., MANNING, L.R. & MANNING, J.M. (1992). Nitric oxide can modify amino acid residues in proteins. *Biochem. Biophys. Res. Commun.*, **183**, 598–604.

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.

RADOMSKI, M.W., REES, D.D., DUTRA, A. & MONCADA, S. (1992). S-nitroso-glutathione inhibits platelet activation *in vitro* and *in vivo*. *Br. J. Pharmacol.*, **107**, 745–749.

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.

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.

TAKEMA, M., INABA, K., UNO, K., KAKIHARA, K.-I., TAWARA, K. & MURAMATSU, S. (1991). Effect of L-arginine on the retention of macrophage tumoricidal activity. *J. Immunol.*, **146**, 1928–1933.

WHITE, K.A. & MARLETTA, M.A. (1992). Nitric oxide synthase is a cytochrome P-450 type hemoprotein. *Biochemistry*, **31**, 6627–6631.

XIE, Q., CHO, H.J., CALAYCAY, J., MUMFORD, R.A., SWIDEREK, K.M., LEE, T.D., DING, A., TROSO, T. & NATHAN, C. (1992). Cloning and characterization of inducible nitric oxide synthase from mouse macrophages. *Science*, **256**, 225–228.

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