Nicotinamide inhibits nitric oxide synthase mRNA induction in activated macrophages

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Nitric oxide (NO) is a potent mediator involved in many biological functions including inflammation and non-specific immunity. Murine macrophages possess the prototype of high-output NO synthase which is not constitutively expressed but induced within a few hours by immunological stimuli. In this study, we explored the possibility of controlling the activity of the inducible NO synthase by interfering with the transduction signal which triggers its induction, in the RAW 264.7 macrophage cell line. We found that nicotinamide, an inhibitor of ADP-ribosylation, prevented NO synthase induction in RAW 264.7 cells after stimulation with interferon γ (IFN- γ) and lipopolysaccharide (LPS). Furthermore, the level of NO synthase mRNA was measured by Northern-blot analysis and we found that

nicotinamide prevents expression of NO synthase mRNA in IFN- γ - and LPS-stimulated cells. Nicotinamide was also found to inhibit other macrophage functions expressed in response to IFN- γ , i.e. tumour necrosis factor secretion and the expression of the Ia antigen of the major histocompatibility complex. Analysis of the pattern of ADP-ribosylated proteins revealed that nicotinamide as well as cholera toxin prevented the ADP-ribosylation of a 107–117 kDa protein found constitutively ADP-ribosylated in stimulated and non-stimulated macrophage extracts. Together, our results indicate ADP-ribosylation as a crucial point of the signalling pathway which leads to NO synthase mRNA induction.

INTRODUCTION

L-Arginine-derived nitric oxide (NO) is a mediator produced in mammalian cells by two types of NO synthase: a constitutively expressed type, which releases small amounts of NO required for physiological functions, and an inducible type, responsible for a sustained large output of NO, which, beyond a certain threshold, is potentially toxic (see refs. [1] and [2] for reviews). The NO produced in large amounts by the inducible form of NO synthase plays a multifunctional and somewhat paradoxical part in the immune system. Indeed, it behaves like an effector molecule of macrophage and hepatocyte cytotoxicity against intracellular pathogens [3–6], like a mediator of immunosuppression [7–9] and like a pathogenic factor in autoimmunity [10]. Therefore the biosynthesis and dissemination of such a reactive molecule necessarily requires stringent homeostatic control. It is known from *in vitro* and *in vivo* experiments that NO biosynthesis is differently affected by different cytokines. Thus interferon γ (IFN- γ), interleukins 1 and 2 and tumour necrosis factor (TNF) all enhance production of the inducible type of NO synthase [11–14], whereas transforming growth factor β and interleukins 8 and 10 have all been reported to reduce it [14–17]. Consequently, a challenging issue in this field is to achieve control of high-output NO synthase activity without inhibiting constitutive NO synthase activity. However, it appears that the catalytic mechanisms of the two types of enzyme are too similar to envisage the use of selective inhibitors for the high-output NO synthase. The signalling pathway that triggers high-output NO synthase activity has so far been little investigated and not much information is available about the induction on NO synthase gene transcription. Yet the identification of this pathway may make it possible to

define a better way of preventing the harmful effects of excessive NO release.

The inducible NO synthase prototype has been isolated from the RAW 264.7 macrophage cell line [18,19], after stimulation with IFN- γ and lipopolysaccharide (LPS) (both major macrophage activators), and its cDNA has been cloned in this cell line by several laboratories [20–22]. In the present study, we investigated the possibility of modulating the transductional events involved in NO synthase induction in RAW 264.7 macrophages.

As poly(ADP-ribosylation) has been reported to be involved in the expression of certain IFN- γ -inducible genes [23,24], we decided to explore the question of whether inhibitors of ADP-ribosylation inhibit the induction of NO synthase activity in RAW 264.7 cells after their stimulation by IFN- γ and LPS. We found that nicotinamide inhibited nitrite synthesis in these cells by preventing NO synthase mRNA induction, without inhibiting NO synthase activity. Two other important macrophage functions induced by IFN- γ , i.e. TNF secretion and the expression of major histocompatibility complex (MHC) Class II, were also prevented by nicotinamide.

MATERIALS AND METHODS

Media and reagents

Dulbecco's modified Eagle's medium (DMEM) and low-endotoxin fetal bovine serum (FBS) were obtained from Gibco Laboratories (Paisley, Scotland, U.K.). Murine recombinant IFN- γ (specific activity 2×10^7 units/mg) and murine recombinant TNF- α (specific activity 3×10^7 units/mg) were from Genentech (South San Francisco, CA, U.S.A.) and were kindly provided by Dr. G. R. Adolf (Boehringer-Mannheim, Vienna,

Abbreviations used: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PARP, poly(ADP-ribose)polymerase; IFN- γ , interferon γ ; LPS, lipopolysaccharide; TNF, tumour necrosis factor; MHC, major histocompatibility complex; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum.

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Austria). IFN- γ contained less than 0.016 unit/mg endotoxin according to the supplier. Phenol-extracted LPS from *Escherichia coli*, bacterial toxins and all other chemicals were purchased from Sigma. [32 P]NAD $^{+}$ (specific radioactivity 29.6 TBq/mmol) and [α - 32 P]dCTP (specific radioactivity 111 TBq/mmol) were obtained from Dupont de Nemours (NEN-France, Les Ulis, France).

The murine macrophage cell line RAW 264.7 and the murine fibroblast cell line L929 were purchased from the American Type Culture Collection (Rockville, MD, U.S.A.) and were maintained in DMEM plus 5% FBS.

Nitrite determination

Nitrite was measured spectrophotometrically as previously described [25], using the Griess reagent containing final concentrations of 0.5% sulphanilamide and 0.05% *N*-(1-naphthyl)-ethylenediamine hydrochloride (in 45% acetic acid).

Preparation of soluble extracts and NO synthase activity determination

RAW 264.7 cells were activated by incubation for 15 h with 100 units/ml IFN- γ , alone or with 10 ng/ml LPS. At the end of incubation, RAW 264.7 cell monolayers were washed and scraped off the culture dishes in PBS. Cells were resuspended at a density of 25×10^6 /ml in 10 mM Tris/HCl, pH 7.5, containing the protease inhibitors, aprotinin (5 μ g/ml), pepstatin (5 μ g/ml), soya-bean trypsin inhibitor (10 μ g/ml) and phenylmethanesulphonyl fluoride (0.1 mM). Soluble extracts were prepared as previously described [26]; briefly, cell suspensions were sonicated in an ice bath with two bursts of 10 s (maximum power 40%) with a Vibra Cell sonifier (Bioblock Scientific, Illkirch, France). The lysates were centrifuged for 20 min at 150 000 *g* and 4 °C in a TL100 ultracentrifuge (Beckman Instruments). Portions of the supernatants were immediately frozen at -80 °C and stored until use. NO synthase activity was determined as previously described [25], by incubating soluble extracts for 1 h 30 min at 37 °C with 2 mM L-arginine and 2 mM NADPH. Residual NADPH which interferes with Griess reagent was hydrolysed for 15 min at 37 °C with 2.5 units/ml L-glutamate dehydrogenase, 25 mM α -oxoglutaric acid and 150 mM NH $_4$ Cl. Nitrite levels were determined as described above, using the Griess reagent.

Cell induction and RNA extraction

Exponentially growing cells (1×10^6 cells/ml) were stimulated in fresh complete medium for 3–6 h at 37 °C in the presence or absence of IFN- γ (100 units/ml) with or without LPS (10 ng/ml). When indicated, nicotinamide (10 mM) was added 30 min before IFN- γ with or without LPS. Macrophage monolayers were washed with PBS, and lysed in guanidinium isothiocyanate buffer, and total RNAs were extracted as described by Chomczynsky and Sacchi [27]. Successful isolation of undegraded RNA was checked by minigel electrophoresis in the presence of ethidium bromide and by the identification of 28 S and 18 S ribosomal RNA bands.

Northern-blot analysis and cDNA probes

Specific mRNA was detected by Northern-blot analysis. For this purpose, 25–30 μ g of total RNA was denatured with formaldehyde/formamide, submitted to denaturing 1.1% agarose-gel electrophoresis, transferred to Hybond N $^{+}$ mem-

brane (Amersham, Bucks., U.K.) and hybridized with 32 P-labelled cDNA probes under the conditions suggested by the supplier. NO synthase and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNAs were gifts from Dr. C. J. Lowenstein [22] and Dr. P. Fort [28] respectively. cDNA probes were labelled with [α - 32 P]dCTP by the random-primer method (Boehringer-Mannheim). Hybridization signals were visualized by autoradiography; filters were exposed to Hyperfilm-MP (Amersham) with intensifying screens at -70 °C. Ethidium bromide-stained gels and hybridizations with GAPDH cDNA probe were used as controls.

Measurement of TNF activity

TNF activity was measured by the cytotoxic assay on L929 cells, in the presence of 1 μ g/ml actinomycin D. After incubation at 37 °C, the medium was removed and cells were stained with Crystal Violet, by a previously described procedure [29].

Determination by immunofluorescence of MHC Class-II molecule expression

Total immunofluorescence was determined on permeabilized cells. Cells were fixed in 4% *p*-formaldehyde for 1 h at 4 °C, and washed first in 50 mM NH $_4$ Cl and then in PBS. After centrifugation at 250 *g*, cells were resuspended in PBS containing 1% BSA at a density of 1×10^6 /ml, and 100 μ l portions were used for the immunofluorescence assay. Assays were carried out in 96-microwell culture plates (Nunc). Cells were permeabilized in PBS containing 0.025% saponin, 0.1% azide, 1% BSA and 10% murine serum, to saturate Fc receptors. After thorough washing in PBS containing 0.025% saponin, 0.1% azide and 0.1% BSA, pellets were resuspended for 30 min at 4 °C in a solution containing the antibody M5/114 rat isotype IgG-2b specific for Ia molecules, i.e. for haplotypes H-2b, H-2d and H2q [30]. Isotype control rat antibody was IgG-2b, specific for Ly-2 molecules (CD8a). After several washings, cells were resuspended for 30 min at 4 °C in solution containing the antibody DTAF-F(ab) $_2$ specific for heavy and light IgG chains (Jackson Immuno-research Laboratories, West Grove, PA, U.S.A.). After being washed, cells were fixed again in 4% *p*-formaldehyde, and immunofluorescence was measured with a Facscan analyser (Becton Dickinson).

RNA synthesis

RAW 264.7 cells were cultured at a density of 1×10^6 /ml in 1 ml/well in a 24-well culture plate. Cells were pulse-labelled for 1 h with 1 μ Ci of [3 H]uridine and harvested in 300 μ l of 0.2% Triton X-100 with a rubber policeman; 60 μ l was spotted on 3 MM filter paper (Whatman) and counted for radioactivity after classical trichloroacetic acid treatment. Controls were untreated cells.

ADP-ribosylation in RAW 264.7 cell soluble extracts

Portions of soluble extract (50–80 μ g of protein) were incubated for 1 h at 37 °C in 0.1 M Hepes, pH 7.4, buffer containing 1 μ M NAD $^{+}$ and 1.1 μ Ci of [32 P]NAD (37 kBq) with or without 2 mM cysteine/HCl, pH 7.4, prepared daily. After incubation, proteins were precipitated for 45 min at 4 °C with 1 ml of 10% trichloroacetic acid. The precipitates were centrifuged at 10 000 *g* for 20 min at 4 °C and pellets were washed twice with 1 ml of water-saturated diethyl ether and resuspended in 45 μ l of 62.5 mM Tris/HCl, pH 6.8, buffer containing 10% glycerol, 2% SDS,

5% 2-mercaptoethanol and 0.00125% Bromophenol Blue. The resulting preparations were heated at 100 °C for 5 min and loaded on to 10% polyacrylamide gel containing 0.1% SDS. Gels were run at 10 mA in 0.3% Trizma, pH 8.3, containing 1.44% glycine and 0.1% SDS. Migrations were calibrated using peptides of known molecular mass (200, 97, 69, 46, 30, 21 and 14 kDa). Gels were stained in 0.1% Coomassie Blue R-250/10% acetic acid/40% methanol, destained in 10% acetic acid and 40% methanol, and exposed to Hyperfilm-MP with intensifying screens at -80 °C. Radiolabelled polypeptides were visualized by autoradiography.

RESULTS

Effect of ADP-ribosylation inhibitors on induction of NO synthase activity

RAW 264.7 cells were stimulated by 100 units/ml IFN- γ in the presence of nicotinamide or benzamide, two inhibitors of ADP-ribosylation. Measurement of cell nitrite accumulation after 15 h in culture medium showed that both these agents inhibited the nitrite synthesis induced dose-dependently by IFN- γ , with an IC_{50} of 7.5 ± 1.5 mM (Figure 1a). Nicotinic acid, which does not inhibit ADP-ribosylation, failed to prevent nitrite synthesis. As assessed by lactate dehydrogenase release in the culture medium (< 7%), nicotinamide concentrations of up to 30 mM did not affect cell viability.

NO synthase activity was also assessed in soluble extracts of RAW 264.7 cells previously activated by IFN- γ in the presence of increasing concentrations of nicotinamide. When nicotinamide was present in the culture medium during cell activation, NO synthase activity in the soluble extracts decreased in a dose-dependent manner (Figure 1b). In control experiments, nicotinamide concentrations of up to 50 mM did not interfere with NO synthase activity.

LPS also induces NO synthesis in macrophages, both alone and in synergy with IFN- γ [12,15]. As shown in Figure 1(c), nitrite production by RAW 264.7 cells treated with increasing doses of IFN- γ , with or without LPS, was greatly reduced by nicotinamide. Nicotinamide also inhibited nitrite production by cells stimulated by LPS alone (not shown).

Taken together, these results imply that nicotinamide inhibited the induction of NO synthase activity.

Effect of nicotinamide on the expression of NO synthase mRNA in immuno-stimulated macrophages

To further define the effect of nicotinamide, we determined the level of NO synthase mRNA expression in RAW 264.7 cells. For this purpose, cells were treated with IFN- γ and LPS in the presence or absence of nicotinamide, and total RNA was extracted after 3 or 6 h of incubation. Determination of the NO synthase mRNA level by Northern-blot analysis showed that this mRNA was not constitutively expressed, and that it was already detectable as early as 3 h after stimulation (Figure 2, lane D). When nicotinamide was present in the culture medium during cell activation, and RAW 264.7 cells were activated with IFN- γ and LPS, little or no NO synthase mRNA was detected (Figure 2, lanes F and K). When cells were activated with IFN- γ alone, only a faint band was detectable when the film was overexposed, and only after 6 h (not shown). When nicotinamide was added to cells together with IFN- γ , no mRNA was detected either (Figure 2, lane J). IFN- γ , with or without LPS or nicotinamide, did not modify the GAPDH mRNA level (Figure 2). Further, after 6 h of treatment with 10 mM nicotinamide, the

latter did not reduce labelled uridine incorporation by RAW 264.7 cells: $100 \pm 5\%$ of control (mean $\pm$ S.E.M. of four independent experiments). Taken together, these data indicate that nicotinamide did not lower the overall level of RNA expression under our experimental conditions.

Effect of nicotinamide on TNF secretion by RAW 264.7 cells

TNF was previously reported to be an autocrine cofactor required for NO synthesis induction in activated macrophages [11]. Therefore we wondered whether nicotinamide could inhibit TNF

Figure 1 Effect of ADP-ribosylation inhibitors on NO synthase activity in stimulated RAW 264.7 cells

(a) Cells in culture (1×10^6 /ml) were stimulated by incubation for 15 h with 100 units/ml IFN- γ in the presence of increasing concentrations of nicotinamide ($\square$), benzamide ($\blacksquare$) or nicotinic acid ($\triangle$), added 1 h before IFN- γ . (Nitrite levels were then determined in culture supernatants and expressed as % of control (IFN- γ alone); 100% represents 15 nmol of nitrite/ 10^6 cells. Results are means $\pm$ S.D. of three independent experiments. (b) At the end of the incubation, cell extracts were prepared as described in the Materials and methods section, and NO synthase activity was determined as nmol of nitrite produced/mg of cell extract in 1.5 h and expressed as % of control (cell extract activity of cells stimulated by IFN- γ alone). Results are means $\pm$ S.D. of three independent experiments. (c) RAW 264.7 cells were stimulated by incubation for 15 h with increasing concentrations of IFN- γ alone ($\blacksquare$) or with 10 ng/ml LPS ($\blacktriangle$). When indicated, 10 mM nicotinamide (N) was added 1 h before IFN- γ ($\square$) or IFN- γ + LPS ($\triangle$). Nitrite levels were determined in culture supernatants at the end of the incubation. A representative experiment of three is shown.

Figure 2 Prevention by nicotinamide of NO synthase mRNA induction

Total RNA (25 µg) from RAW 264.7 cells treated with the indicated inducers for 3 or 6 h were subjected to Northern-blot analysis. The blot was hybridized with an NO synthase probe, as described in the Materials and methods section. Hybridization with a GAPDH probe was performed on the same blot after NO synthase hybridization, without prior dehybridization. IFN-γ is 100 units/ml; LPS, 10 ng/ml; nicotinamide, 10 mM.

Table 1 Prevention by nicotinamide of the TNF secretion induced by IFN-γ and LPS

RAW 264.7 cells (0.4×10^6 cells/ml) were treated for 2, 3, or 6 h with 100 units/ml IFN-γ plus 10 ng/ml LPS. When indicated, nicotinamide (10 mM) was added 1 h before IFN-γ and LPS. TNF activity was determined by a biological assay as described in the Materials and methods section. A representative experiment of two is shown.

Additives	TNF (units/ml)					
	— Nicotinamide			+ Nicotinamide		
	2 h	3 h	6 h	2 h	3 h	6 h
IFN-γ + LPS	8	32	256	< 2	4	32

Table 2 Failure of TNF addition to restore nitrite synthesis inhibited by nicotinamide

Data are from the experiment illustrated in Figure 1. Nicotinamide (10 mM) was added 1 h before IFN-γ (200 units/ml) or IFN-γ plus LPS (10 ng/ml), whereas TNF-α (2000 units/ml) was added 1 h after IFN-γ or IFN-γ plus LPS. Results from a representative experiment of two are shown.

Cell treatment	NO ₂ ⁻ (µM)	
	— TNF-α	+ TNF-α
None	0	0
IFN-γ	6	14
IFN-γ + nicotinamide	2	6
IFN-γ + LPS	57	57
IFN-γ + LPS + nicotinamide	31	33

secretion and, consequently, prevent NO synthase induction. Firstly, we determined the TNF level in RAW 264.7 cell supernatants by measuring its toxicity on L929 fibroblasts in the presence of actinomycin D [29]. A typical experiment, illustrated in Table 1, indicated that nicotinamide did prevent the TNF secretion jointly induced by IFN-γ and LPS. When cells were

Figure 3 Immunofluorimetric analysis of the modulation of MHC Class-II molecules in RAW 264.7 cells

Representative graphs of the specific fluorescence of Ia molecules in saponin-permeabilized RAW 264.7 cells are shown. Specific fluorescence intensities were detected by indirect immunofluorescence, as described in the Materials and methods section. Cells were treated for 21 h with 200 units/ml IFN-γ alone (C), or combined with 10 mM nicotinamide (B). Curve A, control showing the immunofluorescence obtained with anti-CD8 antibody in IFN-γ-activated cells. Results are expressed as the relative number of cells (y-axis) versus log fluorescence intensity (x-axis).

activated by IFN-γ alone, weak TNF activity of 16–32 units/ml was detectable, but only after 15–18 h. Whatever the stimulant used, no TNF activity was detectable in the presence of nicotinamide. Nicotinamide (10 mM) did not inhibit lysis of L929 cells in the presence of TNF-α and actinomycin D.

We then explored the question of whether prevention of TNF secretion by nicotinamide is responsible for inhibition of NO synthase induction. For this purpose, we added exogenous TNF to RAW 264.7 cells in an attempt to counteract the blockade by nicotinamide of nitrite synthesis on stimulation by IFN-γ and LPS (Table 2). As expected, TNF-α alone was unable to induce nitrite synthesis, but synergized with IFN-γ in this respect, in agreement with previous results [11,12]. However, the addition of TNF-α to the stimulated cells did not avoid the inhibitory effect of nicotinamide on nitrite production. These results imply that the effects of nicotinamide action cannot be solely explained by its inhibition of TNF-α secretion.

Effect of nicotinamide on MHC Class-II expression in RAW 264.7 cells

As the expression of MHC Class II is another important macrophage function enhanced by IFN-γ [31], we tested the effects of nicotinamide on this expression. RAW 264.7 cells did not constitutively express the Ia antigen of MHC Class II molecules, but when they were treated with IFN-γ, we observed the induction of Ia molecule expression, as measured by indirect fluorescence (Figure 3). Much of this induction was prevented when nicotinamide was added to the culture medium. The number of positive cells dropped from 23 to 2% and from 35 to 11% after 21 and 35 h (not shown) of IFN-γ treatment respectively. These results show that in addition to inhibiting NO synthase induction, nicotinamide also prevents the induction by IFN-γ of MHC Class-II molecules in murine macrophages.

Figure 4 Autoradiography of ADP-ribosylated polypeptides in RAW 264.7 cell extracts

Cell extracts (80 μ g) were prepared from unstimulated control cells (lanes A–C) or from cells stimulated for 6 h with 200 units/ml IFN- γ plus 10 ng/ml LPS (lanes D–H). ADP transfers were performed using [32 P]NAD in a HEPES buffer, as described in the Materials and methods section. Cell extracts were incubated alone (lanes A, D and G), with 1 μ g of *B. pertussis* toxin (PTX) (lanes C and E), with 1 μ g of cholera toxin (CTX) (lanes B and F) or with 20 mM nicotinamide (lane H). Molecular markers (kDa) of migration are indicated.

ADP-ribosylation in RAW 264.7 cells

Nicotinamide has been reported to inhibit ADP-ribosylation, particularly poly(ADP-ribosylation) [32,33]. Furthermore, Berton et al. [24] observed that protein poly(ADP-ribosylation) increased when macrophages were activated by IFN- γ . Taken together with our results, these observations led us to suspect that nicotinamide might inhibit the poly(ADP-ribosylation) required in macrophages for the induction of NO synthase by IFN- γ and LPS.

Accordingly, RAW 264.7 cells were stimulated with IFN- γ and LPS, and the cell-soluble extracts prepared after 6 h were analysed for *in vitro* ADP-ribosylation. Soluble extracts were incubated with [32 P]NAD and parallel tests were conducted with nicotinamide and cholera or *Bordetella pertussis* toxins, both of which are known to catalyse ADP-ribosylation.

As shown in Figure 4, autoradiography of the pattern of labelled products after SDS/PAGE revealed that, in both stimulated and unstimulated cells, two proteins were ADP-ribosylated: one of 37–39 kDa and the other of 107–117 kDa. The labelling of the 37–39 kDa protein was not modified by treatment with nicotinamide or toxins. However, the highly constitutive ADP-ribosylation of the 107–117 kDa polypeptide was inhibited by cholera toxin (Figure 4, lanes B and F) and nicotinamide (Figure 4, lane H), but not by *B. pertussis* toxin (Figure 4, lanes C and E). Inhibition by nicotinamide as well as the width of the band and the intensity of the labelling suggest that the 107–117 kDa protein was poly(ADP-ribosylated).

DISCUSSION

Little is known about the signal-transduction pathway by which immunological stimuli induce NO synthase activity, although Severn et al. [34] recently indicated that protein kinase C may be involved in the induction of NO synthase by IFN- γ . Besides, Hauschildt et al. [35] showed that ADP-ribosylation inhibitors suppress nitrite production by LPS-stimulated macrophages. In

the present work, we showed that nicotinamide and benzamide, two ADP-ribosylation inhibitors, prevented the induction, by IFN- γ used alone or along with LPS, of NO synthase activity in RAW 264.7 macrophages. We found evidence that the prevention of this induction in turn inhibits NO synthesis, because there was almost no expression of NO synthase mRNA when RAW 264.7 cells were stimulated in the presence of nicotinamide. This action of nicotinamide does not seem to affect the overall RNA level, as no modification was observed in GAPDH mRNA expression, and incorporation of labelled uridine was not reduced by 10 mM nicotinamide within the time frame of our study.

These results agree with recent reports by Hauschildt et al. [35,36] showing that nicotinamide suppressed nitrite production by LPS-stimulated macrophages and TNF-treated fibroblasts. However, according to these authors, the mechanism might involve the inhibition of the overall RNA synthesis which we found was not affected by nicotinamide in RAW 264.7 cells. These apparent contradictions can be explained by the fact that we only used 10 mM nicotinamide in this study, whereas Hauschildt et al. [35] observed a significant decrease in uridine uptake when using 20 mM nicotinamide and above.

It is worthy of note that macrophages produce TNF in response to IFN- γ and/or LPS, and we previously showed that anti-TNF antibodies prevent, at least in part, the production of nitrite by activated macrophages [11]. Endogenous TNF thus plays an important part in macrophage NO synthase induction. This feature is in keeping with the fact that exogenous TNF did not exhibit any additive effect to that of IFN- γ and LPS on nitrite production. Hauschildt et al. [36] further showed that nicotinamide inhibits nitrite production by TNF-stimulated L929 fibroblasts. Here therefore we explored the possibility that the action of nicotinamide is mediated by the inhibition of TNF secretion. Our results indicate that, although nicotinamide inhibited the TNF secretion induced by IFN- γ and LPS, the addition of exogenous TNF- α failed to bypass this blockade. Accordingly, inhibition of endogenous production of TNF by stimulated macrophages does not seem to be primarily responsible for the effects of nicotinamide on NO synthase induction. Our results rather support the hypothesis that nicotinamide targets intracellular messenger(s) involved in signal transduction. Nicotinamide is an inhibitor of ADP-ribosylation, and more specifically of poly(ADP-ribose) polymerase (PARP) [32,33]. When we studied the pattern of ADP-ribosylated proteins in RAW 264.7 soluble extracts, we detected two polypeptides which were covalently radiolabelled after *in vitro* ADP-ribosylation: one was a 37–39 kDa protein insensitive to nicotinamide, and the other, a 107–117 kDa protein sensitive to nicotinamide. We observed the same protein pattern in non-activated control cells and in activated cells.

A 39 kDa polypeptide was recently purified and identified as the glycolytic enzyme, GAPDH, in neurons [37], platelets [38], erythrocytes [39] and hepatocytes [40]. Note that NO enhances the endogenous ADP-ribosylation of GAPDH, which leads to inhibition of its enzyme activity [38,40]. We observed here that the 37–39 kDa protein which was ADP-ribosylated in RAW 264.7 cells had the same characteristics as that described in platelets and neurons [41,42], i.e. enhanced ADP-ribosylation by nitroprussate, which was only induced when cysteine or dithiothreitol was added, inhibition by *N*-ethylmaleimide and no modification by nicotinamide (C. Pellat-Deceunynck, J. Wietzerbin and J.-C. Drapier, unpublished work).

As regards the 107–117 kDa protein, we observed that both nicotinamide and cholera toxin, but not *B. pertussis* toxin, inhibited its ADP-ribosylation in unstimulated cells as well as in stimulated cells. Because nicotinamide chiefly inhibits poly(ADP-

ribosyl)ation and because of the width of the band and intensity of the labelling, it is likely that the 107–117 kDa protein was poly(ADP-ribosyl)ated. Cholera toxin and *B. pertussis* toxin generally catalyse a mono(ADP-ribosyl)ation reaction, whereas nicotinamide inhibits the poly(ADP-ribosyl)ation [43]. Therefore the fact that both nicotinamide and cholera toxin induce inhibition of ADP-ribosylation of the 107–117 kDa protein is puzzling and deserves further attention. The interpretation of this observation may be debated but, as a viewpoint, we propose the following hypothesis. The best characterized enzyme so far identified as capable of catalysing poly(ADP-ribosyl)ation is PARP, a 100–130 kDa protein localized mainly in the nucleus [43,44], although this enzyme activity has also been found in extranuclear compartments [43]. PARP plays a role in various processes, including DNA repair, differentiation and gene expression [44]. When activated, it converts itself into a protein carrying multiple ADP-ribose polymers, whereas nicotinamide and aminobenzamide respectively inhibit its activity [32,43] and its autopoly(ADP-ribosyl)ation [45]. Recently, Martinez et al. [46] showed that PARP is mono(ADP-ribosyl)ated in the DNA-binding domain by a reaction catalysed by cholera toxin. Consequently, as suggested by the authors, it is reasonable to expect that this biochemical modification could lead to inhibition of PARP activity including its autopoly(ADP-ribosyl)ation. Even though the following hypothesis remains to be demonstrated experimentally, it is tempting to speculate that the 107–117 kDa polypeptide, which was ADP-ribosylated in RAW 264.7 macrophages and sensitive to both cholera toxin and nicotinamide, is in fact PARP. Our findings and these considerations allow us to hypothesize that nicotinamide might interfere with the intracellular signalling pathway involved in NO synthase induction by inhibiting PARP activity.

In conclusion, our results shed light on the transductional mechanism involved in NO synthase transcription and allow us to propose that the down-modulation of ADP-ribosylation might help to control excess NO production in certain pathological situations.

Note added in proof (received 4 November 1993)

After this paper was accepted for publication, McDonald and Moss [47] reported that ADP-ribosylation of GAPDH is actually an NAD⁺ linkage.

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