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Induction of nitric oxide synthase activity in phagocytic cells inhibited by tricyclodecan-9-yl-xanthogenate (D609)

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1 The synthesis of nitric oxide (NO) by immune-stimulated murine phagocytic cells (J774) and the modulation of this synthesis by tricyclodecan-9-yl-xanthogenate (D609), a specific inhibitor of phosphatidylcholine-specific phospholipase C (PC-PLC), was investigated. D609 dose-dependently suppressed production of NO, as measured by the release of nitrite and nitrate, in response to lipopolysaccharide (LPS) and interferon-γ (IFN-γ) in intact cultured cells with an IC_{50} of approximately 20 μg ml^{-1}. D609 at 40 μg ml^{-1} completely abrogated immune-stimulated nitrite production.

2 The inhibitory effects of D609 on nitrite production were time-dependent and restricted to the first 18 h post-stimulation. D609 did not inhibit nitrite production in the cytosol of immune-stimulated phagocytes.

3 These findings indicate that the xanthogenate, D609, is a potent inhibitor of the induction of NO-synthase activity in immune-stimulated phagocytes. Furthermore, since D609 has been demonstrated to inhibit PC-PLC specifically, our findings suggest that the activation of this enzyme by LPS and IFN-γ is a proximal step in the signal transduction of inducible NO-synthase in phagocytic cells.

Keywords: Interferon-γ; lipopolysaccharide; nitrate; nitric oxide (NO); nitrite; murine macrophages, phospholipase C; protein kinase C; signal transduction; xanthogenates

Introduction

Nitric oxide (NO) has been recognized as an important mediator of various cellular activities. For example, NO regulates vascular tone and blood flow (Palmer *et al.*, 1987) and in sepsis, excess production of NO by macrophages and other cells activated by endotoxin and cytokines, is thought to induce massive vasodilatation and shock (Kilbourn *et al.*, 1990; Meyer *et al.*, 1992). Moreover, NO synthesized by macrophages contributes to their cytotoxic activity against bacteria (Granger *et al.*, 1990). NO reacts rapidly with water and oxygen to yield nitrite and nitrate, the accumulation of which provides a measure of NO production (Stuehr *et al.*, 1989).

As identified by molecular cloning, there are at least three distinct classes of nitric oxide synthases (NOS) producing NO. Two of them are dependent upon calcium/calmodulin and are constitutively expressed in rat brain (Bredt *et al.*, 1991) and in bovine and human endothelial cells (Lamas *et al.*, 1992; Marsden *et al.*, 1992), respectively. The other type of NOS (termed iNOS) is a Ca^{2+}-independent enzyme and is induced in macrophages, smooth muscle cells, hepatocytes, and fibroblasts several hours after exposure to some cytokines and endotoxin (LPS) (Moncada *et al.*, 1991).

To date, iNOS from murine macrophages has been cloned and characterized (Xie *et al.*, 1992), but little is known about the intracellular signal transduction mechanisms involved in the expression of iNOS. Glucocorticoids have been found to inhibit iNOS expression in activated endothelial cells (Radomski *et al.*, 1990). In addition, cytokines such as transforming growth factor-β, interleukin-4 (IL-4), macrophage deactivating factor, and IL-10 were shown to inhibit iNOS activity induced by interferon-γ (IFN-γ) but had no inhibitory effect on the NO release induced by IFN-γ plus lipopolysaccharide (LPS) (Ding *et al.*, 1990; Oswald *et al.*, 1992). Recently, inhibitors of nuclear transcription factor kappa B (NF-kB) and inhibitors of protein kinase C (PKC), a regulatory enzyme known to activate NF-kB (Gosh & Baltimore, 1990), have been shown to suppress the induction of iNOS activity in LPS- and IFN-γ-stimulated macrophages (Severn *et al.*, 1992; Mülsch *et al.*, 1993; Sherman *et al.*, 1993; Tschaikowsky *et al.*, 1994). These findings suggest that expression of iNOS is regulated by NF-kB.

We questioned, whether there is also a common signal proximal in the induction pathway of iNOS, mediating activation of NF-kB and subsequent synthesis of iNOS activity. Phosphatidylcholine-specific phospholipase C (PC-PLC) has recently been demonstrated to control the upregulaton of NF-kB in response to TNF (Schütze *et al.*, 1992). In these experiments, tricyclodecan-9-yl-xanthogenate (coded D609), previously described as an antiviral (Sauer *et al.*, 1984) and antitumoural (Amtmann & Sauer, 1990) compound, has been demonstrated to inhibit PC-PLC specifically (Schütze *et al.*, 1992). Additionally, D609 has been shown to suppress dose-dependently the TNF-induced release of diacylglycerol (DAG) (Schütze *et al.*, 1992).

In the present study, we investigated the role of D609 in the induction of iNOS activity in immune-stimulated murine macrophages, and discuss the role of PC-PLC and PKC in the signal transduction of iNOS.

Methods

Cell culture and treatment of cells

J774A.1 cells, a mouse macrophage-like cell line (American Type Culture Collection, Rockville, MD, U.S.A.) were grown continuously in 250 ml plastic bottles (Falcon). Supplemented modified Eagle's medium (SMEM) without Phenol red containing 10% heat-inactivated foetal bovine serum Heartline (endotoxin tested <50 pg ml^{-1}) 0.11 g l^{-1} sodium pyruvate, glucose 3.5 g l^{-1}, 100 u ml^{-1}penicillin, 100 μg ml^{-1} streptomycin and 2 mM L-glutamine was used as culture medium. Cells were harvested by centrifugation and then resuspended in culture medium. To induce NO-synthesis, cells were cultured at a concentration of 1×10^6 ml^{-1} in 24 well cluster plates (Costar). Two hours after incubation (37°C, 5% CO_2), medium was aspired to remove non-adherent cells and replaced by fresh, Phenol red-free culture medium adjusted to pH 7.0 (D609 is only active at pH ≤ 7.0), with various

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concentrations of D609 that ranged from 0 (control) to 40 $\mu g\,ml^{-1}$, final concentration. After 1 h of incubation, iNOS activity was induced by adding 1 $\mu g\,ml^{-1}$ LPS (*E. coli* serotype 0111:B4) and 200 u ml^{-1} IFN-γ (final concentrations) either alone or in combination. If not stated otherwise, supernatants were collected at 18 h after stimulation. In subsequent time course experiments, D609 (30 $\mu g\,ml^{-1}$) was added to the cultures at 2, 6, 12 and 18 h after stimulation. In these experiments, cells were incubated (37°C, 5% CO_2) for 24 h, followed by recovery of the supernatant. Supernatants were subsequently analysed for nitrite and nitrate. Some wells were used to estimate the cytotoxic effect of D609 (10–40 $\mu g\,ml^{-1}$), LPS and IFN-γ. Cell viability at the end of the experiments, as assessed by Trypan blue exclusion, exceeded 90% at all concentrations of D609 and under all stimulatory conditions used.

Preparation of cytosol from stimulated macrophages

J774 cells grown in culture bottles were stimulated with LPS (1 $\mu g\,ml^{-1}$) and IFN-γ (200 u ml^{-1}). After 18 h of incubation (37°C, 5% CO_2), cells were harvested by mechanical agitation. After centrifugation (400 *g*, 10 min, 4°C), cells were washed in PBS and resuspended in deionized water containing protease inhibitors (0.1 mM phenylmethyl sulphonylfluoride, 5 $\mu g\,ml^{-1}$ aprotinin, 1 $\mu g\,ml^{-1}$ chymostatin, 5 $\mu g\,ml^{-1}$ pepstatin A; final concentrations). Cells were adjusted to a concentration of $10^7\,ml^{-1}$ and then lysed by three cycles of rapid freezing and thawing. Cell lysates were centrifuged (50,000 *g*, 1 h, 4°C), and aliquots of cytosol were stored at −70°C. Cytosol (70 µl) was incubated with 2 µl NADPH (30 mM), 4 µl L-arginine (30 mM), and 4 µl $MgCl_2$ (30 mM) for 2 h at 37°C. Under these conditions iNOS activity was optimal. For determination of the total amount of accumulated nitrate and nitrite, activated cytosol (80 µl) was further processed as described (nitrate assay). To assess the concentration of nitrite, residual NADPH, which interferes with the Griess reaction, was enzymatically oxidized to $NADP^+$ by addition of 10 µl of a quench solution (25 mM α-ketoglutarate, 0.15 M NH_4Cl, and 0.52 u L-glutamic dehydrogenase from *Proteus* species; final concentrations) (Marletta *et al.*, 1988). Complete oxidation of NADPH to NADP was achieved after 2 h of incubation at 37°C, as determined by the loss of absorbance at 340 nm (data not shown). Finally, 10 µl PBS was added and nitrite was determined as described below.

iNOS activity of the cytosol was defined as the amount of nitrite and nitrate produced per mg of protein and per minute of incubation. In some experiments N^G-monomethyl-L-arginine (0.5–5 mM) was added to the cytosol in order to determine whether the formation of nitrite and nitrate was due to iNOS activity.

Adherent cell protein determination

After complete recovery of the supernatant, remaining cell layers were washed with PBS and lysed in 1 ml PBS containing Triton X-100 (0.02%). Cell lysates were centrifuged (50,000 *g*, 1 h, 4°C) and stored at −20°C. The Pierce bicinchoninic acid method was used to determine protein concentrations, with human serum albumin as standard.

Determination of NO-production

Production of NO in the cell culture was determined by the accumulation of nitrite using a colorimetric assay based on the Griess reaction (Green *et al.*, 1982). Where indicated, nitrate in the sample was additionally measured.

Nitrite assay

Nitrite (NO_2-) concentration in samples of supernatant or activated cytosol was assessed as follows. Griess colour reagent was freshly prepared by combining (1:1) reagent A (0.1% N-(1-naphthyl)ethylenediamine) and reagent B (1%, w/v, sulphanilamide in 5% H_3PO_4). Samples (100 µl) were transferred to a 96 well plate (Costar). Colour reagent (100 µl) was added and after 30 min incubation at 21°C, absorbance was read at 560 nm using a MKII Titertek plate reader. Standard curves were constructed using sodium nitrite (0–120 µM) dissolved in Phenol red-free culture medium.

Nitrate assay

Samples of supernatant (70 µl) together with 2 µl NADPH (30 mM) and 8 µl distilled H_2 or activated cytosol (80 µl) were added per well of a 96 well plate (Costar). Complete reduction of nitrate (NO_3-) to nitrite was achieved by adding 10 µl *Aspergillus* nitrate reductase [EC 1.6.6.2.] and 60 min incubation at 37°C. Residual NADPH was quenched by addition of 10 µl freshly prepared quench solution. Nitrite concentration present before conversion of nitrate to nitrite was determined in parallel by using the same protocol, but adding PBS instead of nitrate reductase. Nitrate concentrations were calculated as difference between nitrite concentrations measured with and without nitrate reductase. Sodium nitrate standards were run in parallel to nitrite standards to verify complete conversion of nitrate to nitrite.

Reagents and media

Tricyclodecan-9-yl-xanthogenate (D609) was kindly provided by Dr G. Sauer, German Cancer Research Center, Heidelberg, Germany. D609 was freshly prepared as a 0.1% stock solution in distilled water and then further diluted in culture medium at pH 7.0. Recombinant mouse IFN-γ was obtained from Genzyme. The 'BCA Protein Assay' kit was purchased from Pierce Chemical Company. Culture medium and all other chemicals were purchased from Sigma.

Statistics

Results are presented as mean ± standard error of the mean (s.e.mean) and were compared by Student's *t* test for unpaired data. Only *P* values <0.05 were considered statistically significant. For multiple comparisons, analysis of variance (ANOVA) was performed. In case of significant *F* values, ANOVA was followed by *t* tests for unpaired data. The Bonferroni correction was used to correct the *t* statistic for the number of comparisons made between groups.

Results

LPS and IFN-γ, either alone or in combination, substantially induced expression of iNOS activity in J774 cells with a subsequent release of NO, as measured as NO_2^- after 18 h of incubation. Inhibition of PC-PLC by D609 added 1 h before stimulation, markedly diminished the production of NO_2^- in the supernatant of cells under all stimulatory conditions tested (Figure 1). However, when D609 was added directly to the activated cytosol of LPS- and IFN-γ-stimulated macrophages, no inhibitory effect on the enzyme activity of iNOS was found. Under the conditions chosen, production rate was 0.12 nmol $NO_2^-\,mg^{-1}$ protein min^{-1} which correlates well with the rate of nitrite production of 0.09 nmol mg^{-1} of protein min^{-1} reported for the cytosol of stimulated RAW 264.7 cells (Marletta *et al.*, 1988). Production rate for the total of NO_2^- and NO_3^-, that was reduced and measured as NO_2^-, was approximately 0.34 nmol $NO_2^-\,mg^{-1}$ protein min^{-1}. Nitrite production by the cytosol of stimulated macrophages was due to iNOS activity, since it could be completely abrogated by incubation with 5 mM NMMA (data not shown).

Therefore, activation of PC-PLC with the subsequent release of DAG seems to be a primary step in iNOS induc-

tion. At the end of each experiment, cell viability was above 90% in all groups. Moreover, adherent cell protein values from 10^6 J774 cells following 18 h of incubation were 149 ± 18 μg in the control group and did not differ significantly from the means of groups treated with D609 (data not shown). These findings indicate that the suppression of iNOS activity by D609 at the concentrations tested, can neither be explained by a cytotoxic nor by an inhibitory effect on cell proliferation. Additionally, the ratio of nitrite to nitrate in the supernatant was invariably about 55% to 45%, whereas in the stimulated cytosol, the ratio was about 35% to 65%, irrespective of the treatment. These ratios of nitrite to nitrate are in close agreement to results of previous studies (Iyengar *et al.*, 1987).

Inhibition of LPS- and IFN-γ-induced NO_2^-/NO_3^- production by D609 was dose-dependent (Figure 2). At 10 μg ml^{-1}, D609 induced significant suppression of NO_2^- production in J774 cells stimulated with LPS (1 μg ml^{-1}) and IFN-γ (200 u ml^{-1}). The IC_{50} value for inhibition of the nitrite release by D609 was approximately 20 μg ml^{-1}. At 40 μg ml^{-1}, added 1 h prior to stimulation, D609 suppressed the induction of iNOS activity by 90–100%.

We next carried out experiments to determine the time at which expression of iNOS activity was susceptible to inhibition by D609. As illustrated in Figure 3, D609 time-dependently inhibited the amount of NO_2^- accumulated after 24 h in the supernatant of stimulated cells. Up to 18 h after stimulation, we observed an almost linear relationship between the time D609 was present in the culture and the amount of inhibition. However, addition of D609 18 h after

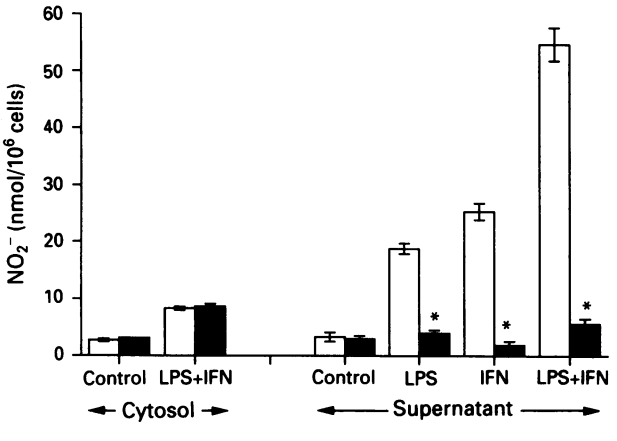

Figure 1 Effect of tricyclodecan-9-yl-xanthogenate (D609) on inducible nitric oxide synthase (iNOS) activity. Data represent the amount of nitrite (NO_2^-) in the supernatant and activated cytosol of stimulated murine J774 macrophages (means ± s.e.mean, $n = 8$) in the absence (open columns) or presence of D609 (30 μg ml^{-1}) (solid columns). Supernatants were collected at 18 h after stimulation. Cytosol prepared from stimulated cells (≈1.49 mg protein from 10^7 cells) was incubated (37°C, 5% CO_2) with L-arginine (1.5 mM), NADPH (0.75 mM) and $MgCl_2$ (1.5 mM) for 2 h. Subsequently, nitrate reductase was added. After 1 h of incubation (37°Ç, 5% CO_2), residual NADPH was enzymatically quenched, and the concentration of NO_2^- was determined. Pretreatment with D609 significantly inhibited NO_2^- production in the supernatant of cells stimulated by lipopolysaccharide (LPS; 1 μg ml^{-1}), interferon gamma (IFN; 200 u ml^{-1}), or both (LPS + IFN). D609 did not affect unstimulated cells (control) or iNOS activity in activated cytosol. *Indicates $P < 0.001$ versus stimulated cells without D609 (*t* test).

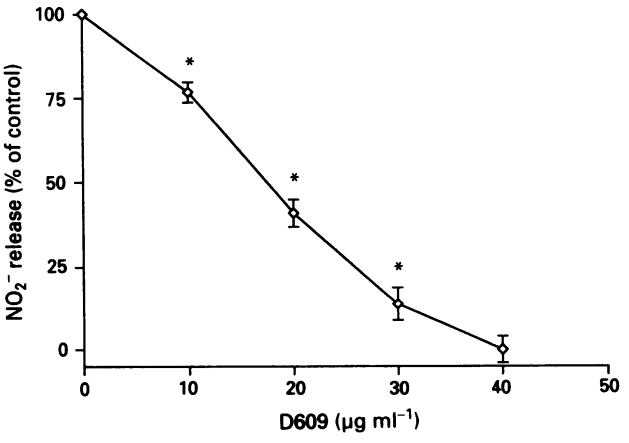

Figure 2 Dose-dependent inhibition of inducible nitric oxide synthase activity by D609. Macrophages were treated with D609 (0–40 μg ml^{-1}) 1 h prior to stimulation with lipopolysaccharide (1 μg ml^{-1}) plus interferon gamma (200 u ml^{-1}). After incubation for 18 h, supernatants were assayed for nitrite. Results are presented as means ± s.e.mean of duplicates from eight different experiments. *Indicates $P < 0.01$ versus mean of cells treated with the next lower concentration of D609 (ANOVA followed by *t* test).

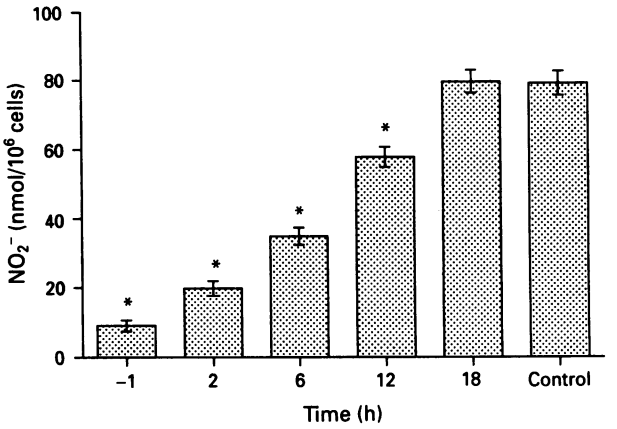

Figure 3 D609 inhibits the induction of nitrite (NO_2^-) synthesis, but not the activity of nitrite producing enzymes. Cells were incubated (37°C, 5% CO_2) with lipopolysaccharide (1 μg ml^{-1}) plus interferon gamma (200 u ml^{-1}) (zero time point) in the absence (control) or presence of D609 (30 μg ml^{-1}) added to the culture at the indicated times (−1 h to 18 h). The data represent the amount of NO_2^- accumulated in the supernatant at 24 h. D609 added at 18 h after stimulation did not reduce the concentration of NO_2^- as compared to control. Importantly, iNOS was still active between 18 h and 24 h, producing approximately 4.5 nmol NO_2^- (10^6 cells)$^{-1}$ h^{-1}). Results are given as means ± s.e.mean of duplicates from eight different experiments. *Indicates $P < 0.001$ versus control (*t* test).

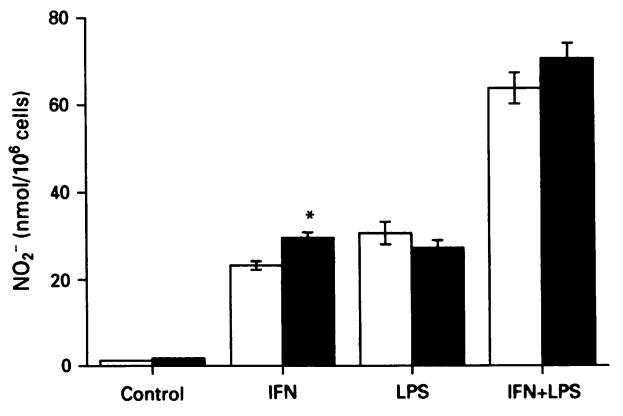

Figure 4 Effect of phorbol 12-myristate 13-acetate (PMA) on inducible nitric oxide synthase (iNOS) activity. Results represent the nitrite (NO_2^-) production (means ± s.e.mean, $n = 4$) in culture supernatant of unstimulated cells (control) and macrophages stimulated for 24 h by lipopolysaccharide (LPS; 1 μg ml^{-1}), interferon gamma (IFN; 200 u ml^{-1}), or both (LPS + IFN), in the absence (open columns) or presence of PMA (1 μg ml^{-1}) (solid columns). *Indicates $P < 0.05$ versus stimulated cells without PMA (*t* test).

the stimulation, did not reduce further accumulation of NO_2^- in the supernatant between 18 h and 24 h. Together with the finding that D609 did not inhibit enzyme activity of iNOS in the activated cytosol, this finding suggests that 18 h after co-stimulation with LPS and IFN-γ, macrophages stopped *de novo* synthesis of iNOS.

We also investigated whether activation of PKC could induce iNOS activity and eventually overcome the inhibitory effect by D609. In these experiments cells were incubated with PMA (1 $\mu g\,ml^{-1}$) that directly activates PKC, but not acidic sphingomyelinase (SM-ase) (Kolesnick, 1987). We found that PMA alone did not induce nitrite production. In combination with other stimuli, PMA enhanced only marginally the induction of iNOS activity by IFN-γ showing no significant effect on NO_2^- production induced by LPS and LPS + IFN-γ (Figure 4). Furthermore, addition of PMA to LPS and IFN-γ could not prevent the suppression of iNOS induction by D609 (data not shown).

Discussion

D609 dose-dependently reduced NO_2^- and NO_3^- production in response to LPS, IFN-γ and a combination of both. The ratio of nitrite to nitrate in the supernatant was unchanged by D609, excluding a shift within the oxidation products of NO. D609 did not inhibit NO production by iNOS present in lysates of stimulated J774 cells and was no longer inhibitory on NO production in cell cultures when added 18 h after stimulation. These findings indicate that D609 inhibits the induction of iNOS in response to LPS and IFN-γ. Induction of iNOS sensitive to D609 inhibition was found to be limited to the first 18 h post-stimulation, which is in close agreement with the kinetics of iNOS induction found by Ding *et al.* (1990).

The concentration of D609 needed for a 50% reduction in NO_2^- release was 20 $\mu g\,ml^{-1}$, which is nearly the same concentration as that needed to inhibit TNF-induced DAG release by 50% in the human monocytic cell line U937 (Schütze *et al.*, 1992). At 30–40 $\mu g\,ml^{-1}$, D609 completely abrogated NO production in response to LPS and IFN-γ. Again, this correlates well with finding of Müller-Decker (1989) showing that D609 at 30 $\mu g\,ml^{-1}$ almost completely blocked the phorbol ester-stimulated DAG release in hamster fibroblasts.

Together with the finding that D609 specifically inhibits PC-PLC, with no effects on phosphatidylinositol-specific PLC, phospholipase A_2 and phospholipase D (Schütze *et al.*, 1992), our results suggest that iNOS induction by LPS and IFN-γ depends on the activation of PC-PLC and the subsequent release of DAG. In a previous study, LPS has been demonstrated to induce hydrolysis of phosphatidyl-inositol-4,5-biphosphate in murine peritoneal macrophages (Pripic *et al.*, 1987).

Collectively, our results suggest that the activation of PC-PLC is a proximal signal in the induction of iNOS. In agreement with data from the literature, we propose the following signaling pathway as a model of iNOS expression: LPS and IFN-γ activate PC-PLC resulting in a release of DAG. DAG is a well-known activator of PKC, which has recently been implicated in iNOS induction in a study showing that iNOS induction can be blocked by inhibition of PKC (Severn *et al.*, 1992). Moreover, DAG released by PC-PLC has recently been shown to activate acidic SM-ase which subsequently produces ceramide (Schütze *et al.*, 1992). Both pathways, via PKC and via ceramide, lead to an activation of NF-kB (Gosh & Baltimore, 1990; Schütze *et al.*, 1992), that finally induces the expression of iNOS, as indicated by two recent studies (Mülsch *et al.*, 1993; Sherman *et al.*, 1993). It seems difficult, however, to determine which of the two pathways is more important for the induction of iNOS. Activation of PKC has been shown to induce activation of phosphatidylcholine-degrading phospholipase C with subsequent generation of DAG (Kolesnick & Paley, 1987; Müller-Decker, 1989). Thus, PKC may act as an endogenous amplifier for the acidic SM-ase-ceramide pathway. The finding that immune-stimulated iNOS activity is suppressed by inhibitors of PKC (Severn *et al.*, 1992; Tschaikowsky *et al.*, 1994), can therefore not provide clear-cut evidence on which is the prevailing pathway of iNOS induction. In our experiments, PMA, that activates PKC, but not acidic SM-ase (Kolesnick, 1987), enhanced only marginally induction of iNOS activity by IFN-γ, but could not induce iNOS activity *per se*, nor did it show a significant effect on iNOS induction by LPS and LPS + IFN-γ in the absence or presence of D609. These findings seem to favour the hypothesis that subsequent to activation of PC-PLC, both acidic SM-ase and PKC activation are necessary for iNOS induction, but neither pathway is sufficient to induce iNOS activity independently.

In conclusion, we have demonstrated that the xanthogenate D609 is a potent inhibitor of the induction of iNOS in immune-stimulated murine macrophages. Furthermore, our findings suggest that the activation of PC-PLC is a proximal step in the signal transduction of iNOS activity. However, clarification of the complete signal transduction pathway leading to iNOS expression awaits further investigations.

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References

AMTMANN, E. & SAUER, G. (1990). Tumor necrosis factor induces necrosis of human carcinoma xenografts in the presence of tricyclodecan-9-yl-xanthogenate and lauric acid. *Int. J. Cancer*, **45**, 1113–1118.

BREDT, D.S., HWANG, P.M., GLATT, C.E., LOWENSTEIN, C., REED, R.R. & SNYDER, S.H. (1991). Cloned and expressed nitric oxide synthase structurally resembles cytochrome P-450 reductase. *Nature*, **351**, 714–718.

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.

GOSH, S. & BALTIMORE, D. (1990). Activation in vitro of NF-kB by phosphorylation of its inhibitor IkB. *Nature*, **344**, 678–682.

GRANGER, D.L., HIBBS, J.B. Jr., PERFECT, J.R. & DURACK, D.T. (1990). Metabolic fate of L-arginine in relation to microbiostatic capability of murine macrophages. *J. Clin. Invest.*, **85**, 264–273.

GREEN, L.A., WAGNER, D.A., GLOGOWSKI, J., SKIPPER, P.L., WISHNOK, J.S. & TANNENBAUM, S.R. (1982). Analysis of nitrate, nitrite, and [^{15}N]nitrate in biological fluids. *Anal. Biochem.*, **1256**, 131–138.

IYENGAR, R., STUEHR, D.J. & MARLETTA, M. (1987). Macrophage synthesis of nitrite, nitrate, and N-nitrosamines: precursors and role of the respiratory burst. *Proc. Natl. Acad. Sci. U.S.A.*, **84**, 6369–6373.

KILBOURN, R.G., JUBRAN, A., GROSS, S.S., GRIFFITH, O.W., LEVI, R., ADAMS, J. & LODATO, R.F. (1990). Reversal of endotoxin-mediated shock by N^G-methyl-L-arginine, an inhibitor of nitric oxide synthesis. *Biochem. Biophys. Res. Commun.*, **172**, 1132–1138.

KOLESNICK, R.N. (1987). 1,2-Diacylglycerols but not phorbol ester stimulate sphingomyelin hydrolysis in GH3 pituitary cells. *J. Biol. Chem.*, **262**, 16759–16762.

KOLESNICK, R.N. & PALEY, A.E. (1987). 1,2-Diacylglycerols and phorbol ester stimulate phosphatidylcholine metabolism in GH3 pituitary cells. *J. Biol. Chem.*, **262**, 9204–9210.

LAMAS, S., MARSDEN, P.A., LI, G.K., TEMPST, P. & MICHEL, T. (1992). Endothelial nitric oxide synthase: molecular cloning and characterization of a distinct constitutive enzyme isoform. *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 6348–6352.

MARLETTA, M.A., YOON, P.S., IYENGAR, R., LEAF, C.D. & WISHNOK, J.S. (1988). Macrophage oxidation of L-arginine to nitrite and nitrate: nitric oxide is an intermediate. *Biochemistry*, **27**, 8706–8711.

MARSDEN, P.A., SCHAPPERT, K.T., CHEN, H.S., FLOWERS, M., SUNDELL, C.L., WILCOX, J.N., LAMAS, S. & MICHEL, T. (1992). Molecular cloning and characterization of human endothelial nitric oxide synthase. *FEBS Lett.*, **307**, 287–293.

MEYER, J., TRABER, L.D., NELSON, S., LENTZ, C.W., NAKAZAWA, H., HERDON, D.N., NODA, H. & TRABER, D.L. (1992). Reversal of hyperdynamic response to continuous endotoxin administration by inhibition of NO synthesis. *J. Appl. Physiol.*, **73**, 324–328.

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

MÜLLER-DECKER, K. (1989). Interruption of TPA-induced signals by an antiviral and antitumoral xanthate compound: inhibition of a phospholipase c-type reaction. *Biochem. Biophys. Res. Commun.*, **162**, 198–205.

MÜLSCH, A., SCHRAY-UTZ, B., MORDVINTCEV, P.I., HAUSCHILDT, S. & BUSSE, R. (1993). Diethyldithiocarbamate inhibits induction of macrophage NO synthase. *FEBS Lett.*, **321**, 215–218.

OSWALD, I., GAZZINELLI, R.T., SHER, A. & JAMES, S.L. (1992). IL-10 synergizes with IL-4 and transforming growth factor-β to inhibit macrophage cytotoxic activity. *J. Immunol.*, **148**, 3578–3582.

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.

PRIPIC, V., WEIEL, J., SOMMERS, S., DIGUISEPPI, J., GONIAS, S.L., PIZZO, S., HAMILTON, T.A., HERMAN, B. & ADAMS, D.O. (1987). Effects of bacterial lipopolysaccharide on the hydrolysis of phosphatidyl-inositol-4,5-biphosphate in murine peritoneal macrophages. *J. Immunol.*, **139**, 526–533.

RADOMSKI, M.W., PALMER, R.M. & MONCADA, S. (1990). Glucocorticoids inhibit the expression of an inducible, but not the constitutive, nitric oxide synthase in vascular endothelial cells. *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 10043–10047.

SAUER, G., AMTMANN, E., MELBER, K., KNAPP, A., MÜLLER, K., HUMMEL, K. & SCHERM, A. (1984). DNA and RNA virus species are inhibited by xanthates, a class of antiviral compounds with unique properties. *Proc. Natl. Acad. Sci. U.S.A.*, **81**, 3263–3267.

SCHÜTZE, S., POTTHOFF, K., MACHLEIDT, T., BERKOVIC, D., WIEGMANN, K. & KRÖNKE, M. (1992). TNF activates NF-kB by phosphatidylcholine-specific phospholipase C-induced 'acidic' sphingomyelin breakdown. *Cell*, **71**, 765–776.

SEVERN, A., WAKELAM, M.J.O. & LIEW, F.Y. (1992). The role of protein kinase C in the induction of nitric oxide synthesis by murine macrophages. *Biochem. Biophys. Res. Commun.*, **188**, 997–1002.

SHERMAN, M.P., AEBERHARD, E.E., WONG, V.Z., GRISCAVAGE, J.M. & IGNARRO, L.J. (1993). Pyrrolidine dithiocarbamate inhibits induction of nitric oxide sythase activity in rat alveolar macrophages. *Biochem. Biophys. Res. Commun.*, **191**, 1301–1308.

STUEHR, D.J., GROSS, S.S., SAKUMA, I., LEVI, R. & NATHAN, C.F. (1989). Activated murine macrophages secrete a metabolite of arginine with the bioactivity of endothelium-derived relaxing factor and the chemical reactivity of nitric oxide. *J. Exp. Med.*, **169**, 1011–1020.

TSCHAIKOWSKY, K., SCHÖNHUBER, F., MEISNER, M. & RÜGHEIMER, E. (1994). Involvement of phosphatidylcholine-specific phospholipase C and protein kinase C in induction of nitric oxide synthase in macrophages. *Int. Care Med.*, **20** Suppl. 1, S52.

XIE, Q.-W., CHO, H.J., CALAYCAY, J., MUMFORD, R.A., SWIDEREK, 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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