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Nitric oxide synthase and cyclo-oxygenase pathways in the inflammatory response induced by zymosan in the rat air pouch

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1 We have studied the participation of nitric oxide (NO) in an animal model of inflammation, the rat air pouch stimulated with zymosan.

2 Saline or zymosan was injected into 6-day rat air pouches at different time points and measurements were made of cell migration, levels of nitrite/nitrate (NO_2^-/NO_3^-), prostaglandin E_2 (PGE_2), leukotriene B_4 (LTB_4) and secretory phospholipase A_2 ($sPLA_2$) in exudates. Nitric oxide synthase (NOS) activity was determined in high speed supernatants from cells present in pouch exudates. Western blot analysis was also performed on these samples.

3 Zymosan injection induced a time-dependent increase in leukocyte infiltration, NO_2^-/NO_3^- levels and cellular NOS activity that reached a peak by 8 h. Western blot analysis showed the same time course for induction of NOS protein. Colchicine administration to rats inhibited cellular infiltration and decreased the levels of NO metabolites and cellular NOS activity zymosan-injected air pouch at 8 h. NOS activity was present in polymorphonuclear leukocytes (PMNs) and monocytes, but not in the lymphocytes present in exudates. This enzyme is calcium-independent and needs NADPH for activity. PGE_2 levels in exudates showed a time course inverse to that of NOS activity and NO metabolites, with maximum levels of PGE_2 observed at 4 h after zymosan injection.

4 Administration of N^G-nitro-L-arginine methyl ester (L-NAME) or aminoguanidine to rats significantly reduced cellular NOS activity, NO_2^-/NO_3^- levels and chemiluminescence, whereas they were without effect on cell migration and degranulation, eicosanoid levels and $sPLA_2$ activity.

5 Treatment of animals with dexamethasone inhibited cellular NOS activity, NO_2^-/NO_3^- levels, chemiluminescence and the increase in the levels of PGE_2 and LTB_4, with only a weak effect on elastase release.

6 Administration of the selective cyclo-oxygenase-2 (COX-2) inhibitor NS398 to rats strongly reduced PGE_2 levels in exudates without affecting NO metabolites or NOS activity at 4 h after zymosan injection.

7 Our data indicate that NOS is induced in the zymosan-stimulated rat air pouch model of inflammation. This enzyme is expressed in the cells migrating into the air pouch and caused an increased production of NO metabolites in exudates. The results also suggest the presence of an earlier phase in which eicosanoids play the main role, with participation of COX-2 activity, and a later phase mediated by NO. The endogenous release of NO does not modify prostaglandin biosynthesis in this *in vivo* model.

Keywords: Zymosan; rat air pouch; inducible nitric oxide synthase; N^G-nitro-L-arginine methyl ester; aminoguanidine; dexamethasone; cyclo-oxygenase-2; NS398

Introduction

Nitric oxide (NO) is formed from L-arginine by nitric oxide synthase (NOS) (for review see Knowles & Moncada, 1994). The constitutive, calcium-dependent isoform (cNOS) is present in endothelial cells (type III or eNOS) and neurones (type I or nNOS). In contrast, the inducible, calcium-independent enzyme (type II or iNOS) is expressed in macrophages and many other cells in the presence of pro-inflammatory cytokines or endotoxins. Neutrophils also appear to produce NO, and the constitutive form of NOS has been isolated from human neutrophils (Bryant *et al*., 1992).

The role of NO and other reactive nitrogen species in inflammation has not been conclusively established, as they may have both pro-inflammatory and anti-inflammatory effects. NO may contribute to the cytotoxic activity of neutrophils by forming secondary species such as peroxynitrite after reaction with reactive oxygen species (Smith, 1994). Products formed from NO can influence the antimicrobial activity of phagocytes. For example, nitrite is bactericidal or inhibits the bactericidal activity of myeloperoxidase and hydrogen peroxide, depending on the experimental conditions (Klebanoff, 1993). On the other hand, NO can control the levels of superoxide by inactivating this anion (McCall *et al*., 1989) and also by direct inhibition of NADPH oxidase activity present in leukocytes (Clancy *et al*., 1992).

Vasodilatation, a feature of acute inflammation, is dependent on NO release for a number of inflammatory mediators like bradykinin, histamine, substance P, etc. (Palmer *et al*., 1987) and could increase oedema formation as well as facilitating leukocyte migration (Wright *et al*., 1989; Sautebin *et al*., 1995). Another important event in the inflammatory response, leukocyte-endothelial cell adhesion in postcapillary venules, can be influenced by NO, as NOS inhibition in endothelial cells provokes microvascular alterations such as an increase in leukocyte adherence and migration and albumin leakage (Kurose *et al*., 1993).

Endogenous NO potentiates inflammatory responses and seems to be involved in both acute and chronic inflammation (Stefanovic-Racic *et al*., 1994). In fact, NO has been implicated as a mediator of inflammation in experimental models including adjuvant arthritis (Ialenti *et al*., 1993; Stefanovic-Racic *et al*., 1994; Connor *et al*., 1995), arthritis induced by streptococcal cell wall fragments (McCartney Francis *et al*., 1993), arachidonic acid-induced paw oedema (Sautebin *et al*., 1995), mustard oil-induced neurogenic inflammation (Lippe *et al*., 1993) or paw oedema (Honoré *et al*., 1995; Salvemini *et al*.,

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1996), pleurisy (Tomlinson *et al*., 1994; Tracey *et al*., 1995), granuloma (Iuvone *et al*., 1994) and air pouch inflammation (Salvemini *et al*., 1995a) induced by carrageenan in the rat. In addition, the participation of NO has also been suggested in a model of chronic granulomatous inflammation in mice (Vane *et al*., 1994). Consequently, NOS inhibitors can exert anti-inflammatory effects on experimental models when there is overproduction of NO, although there are also data showing the failure of NOS inhibitors to modify some parameters of acute inflammatory responses in rats (Medeiros *et al*., 1995; Perretti *et al*., 1995). Interestingly, NO can be generated in response to cytokines in the inflamed joints of patients with rheumatoid arthritis, where it might cause tissue damage (Farrell *et al*., 1992; Grabowski *et al*., 1996). This is demonstrated by the fact that peroxynitrite-derived products nitrate aromatic amino acids and as a result 3-nitrotyrosine is present in synovial fluid and blood serum of arthritic patients (Kaur & Halliwell, 1994).

Prostaglandins are inflammatory mediators generated by the enzyme cyclo-oxygenase (COX). The constitutive enzyme (COX-1) is responsible for the normal production of prostaglandins in the digestive tract, kidney and elsewhere, while the inducible isoform (COX-2) is upregulated in inflamed tissues and leads to an enhanced production of prostaglandins (Vane *et al*., 1994; Masferrer *et al*., 1994; Anderson *et al*., 1996). Different interactions between NOS and COX pathways can exist and therefore the role of NO in inflammation could depend not only on its direct effects but also on its modulatory influence of prostaglandin biosynthesis. In this regard, there are controversial data about the involvement of NO and COX-2 in the inflammatory process (Salvemini *et al*., 1993; 1995a, b; Vane *et al*., 1994; Swierkosz *et al*., 1995; Sautebin *et al*., 1995).

To evaluate the participation of NO in inflammatory responses we have used the rat air pouch inflamed with zymosan, which injected into specific tissues *in vivo* acts as a phagocytic stimulus inducing a response characterized by a marked release of complement-derived peptides (Konno & Tsurufuji, 1983), eicosanoids and cytokines (Ferrándiz & Foster, 1991). We have demonstrated the induction of NOS by zymosan in this model of inflammation and investigated the relationship between the NOS and COX pathways.

Methods

Rat air pouch

Female Wistar rats weighing 140–160 g were used. Air cavities were produced by subcutaneous injection of 20 ml of sterile air into the back. Three days later 10 ml of sterile air was injected into the cavity (Edwards *et al*., 1981). Six days after the initial air injection, 1 ml of sterile saline or 1 ml of 1% (w/v) zymosan in saline was injected into the air pouch. In the 4 h zymosan-injected air pouch, the NOS inhibitors N^G-nitro-L-arginine methyl ester (L-NAME) and aminoguanidine (200 mg kg^{-1}) and the cyclo-oxygenase-2 inhibitor NS398 (1 mg kg^{-1}) were administered i.p. 30 min before the zymosan injection. Dexamethasone (2 mg kg^{-1}) was administered i.p. 1 h before zymosan. In the 8 h zymosan-injected air pouch, L-NAME and aminoguanidine (200 mg kg^{-1}, i.p.) were administered 30 min before zymosan injection and 4 h after zymosan the animals received an additional dose of 100 mg kg^{-1} by the same route. Dexamethasone (2 mg kg^{-1}, i.p.) was administered 1 h before zymosan and 4 h after the inflammatory stimulus the same dose was repeated. In another set of experiments, animals received colchicine (1.5 mg kg^{-1}, i.p.) 15 min before the injection of zymosan. At different time intervals after the injection of saline or zymosan into the air pouch, rats were killed by cervical dislocation and the exudate was collected in 1 ml of saline. The number of leukocytes present in the exudates was determined with a Coulter Counter, and differential counting was also performed. Cells were pelleted by centrifugation at 1,200 *g* for 10 min at 4°C, and the supernatants were used to measure prostaglandin E_2 (PGE_2) or leukotriene B_4 (LTB_4) levels by radioimmunoassay (Moroney *et al*., 1988), nitrite/nitrate levels and enzymatic activities. The cell pellet was used to measure NOS activity and for Western blotting. Protein was quantified by the Bradford technique (Bradford, 1976) with bovine serum albumin (BSA) as standard.

Quantitation of nitrite (NO_2^-)/nitrate (NO_3^-)

Nitrite was assayed fluorimetrically in microtiter plates according to the method described by Misko *et al*. (1993). The amount of nitrite was obtained by extrapolation from a standard curve with sodium nitrite as a standard. The amount of nitrite + nitrate was determined by the same assay performed in the presence of nitrate reductase and expressed as nitrite equivalents.

NOS assay

Cell pellets obtained by centrifugation of exudates at 1,200 *g* for 10 min at 4°C were resuspended in 10 mM HEPES pH 7.4 containing sacarose (0.32 M), EDTA (100 μM), dithiothreitol (1 mM), phenylmethylsulphonyl fluoride (1 mg ml^{-1}) and leupeptin (10 μg ml^{-1}) (Knowles *et al*., 1990) and sonicated (3×10 s) at 4°C in an ultrasonicator at maximum potency. The resulting homogenate was centrifuged at 1,200 *g* for 10 min at 4°C, followed by centrifugation of the supernatant at 100,000 *g* for 100 min at 4°C. NOS activity was determined in supernatants by monitoring the conversion of L-[^{3}H]-arginine to L-[^{3}H]-citrulline, as described by Mitchell *et al*. (1991). Samples (40 μg protein) were incubated at room temperature for 30 min with 100 μl of the above buffer in the presence of NADPH (1 mM) and a mixture of unlabelled and L-[^{3}H]-arginine (10 μM, 1 μCi ml^{-1}). Incubations were terminated by the addition of 20 mM HEPES (1 ml, pH 5.5) containing 1 mM EGTA and 1 mM EDTA. L-[^{3}H]-citrulline was separated from arginine by adding 1.5 ml of 1 : 1 suspension of Dowex (50W) in water. Radioactivity was measured in supernatants by liquid scintillation counting. Experiments performed in the absence of NADPH determined the extent of L-[^{3}H]-citrulline formation independent of specific NOS activity. Experiments in the presence of NADPH, without calcium and with EGTA (100 μM), determined the calcium-independent (inducible) NOS activity. In another set of experiments, leukocytes present in exudates from 8 h zymosan-injected air pouches were separated by Ficoll-hypaque sedimentation. The cell gradient mixture was centrifuged at 400 *g* for 40 min at 20°C to obtain a pellet (95% pure neutrophils) and a monocyte and lymphocyte layer which was removed and pelleted by centrifugation. The cell pellet was resuspended in RPMI-1640 media pH 7.4 with 10% foetal bovine serum and 2 mM L-glutamine and was incubated at a cell density of 10^7 ml^{-1} in 60/15 mm tissue culture dishes. The cells were allowed to adhere for 2 h at 37°C in a 5% CO_2 atmosphere incubator. The non-adherent cells (80% lymphocytes) were removed by suction of the media followed by two washes with 1 ml of RPMI-1640 and adherent cell fractions (90% monocytes) were collected. Every main cellular fraction of the air pouch exudate, polymorphonuclear leukocytes (PMNs), monocytes and lymphocytes, was sonicated and treated as indicated above to measure NOS activity.

Western blot analysis

High speed supernatants from leukocytes present in pouch exudates were obtained as indicated for NOS activity. Equal amounts of protein (50 μg) were loaded on 7.5% polyacrylamide-SDS gels and transferred onto nitrocellulose membranes for 3 h at 0.25 A. Membranes were blocked for 1 h in phosphate buffered saline (PBS)-Tween 20 (20 mM KH_2PO_4, 0.15 M KCl, 0.3% v/v Tween 20, pH 7.5) containing 2% (w/v) BSA and incubated for 1 h with mouse anti-iNOS polyclonal antibody diluted 1/1,000 in PBS-Tween 20. Blots were washed with PBS-conjugated rabbit antimouse IgG (1/2,000). After

another wash (5 × 10 min) with PBS-Tween 20, the immunoreactive bands were visualized with 3-3′-diaminobenzidine as substrate. Densitometry was performed in a LKB 2202 Ultrascan laser densitometer.

Phospholipase A_2 assay

Phospholipase A_2 was assayed with [^{3}H]-oleate labelled membranes of *Escherichia coli*, following a modification of the method of Franson *et al*. (1974) (Payá *et al*., 1996).

Chemiluminescence

After collection of exudates from 8 h zymosan-stimulated rat air pouches, aliquots of cells (2.5×10^6 ml^{-1}) were immediately mixed with luminol (100 μM) in a total volume of 200 μl and the chemiluminescence was recorded in a Microbeta Trilux counter (Wallac, Turku, Finland) after 4 min incubation at 37°C, previously selected as the time for maximal chemiluminescence. Another series of aliquots were stimulated with 12-*O*-tetradecanoyl phorbol 13-acetate (TPA, 1 μM) at the time of luminol addition.

Elastase assay

After centrifugation of exudates from 8 h zymosan-stimulated rat air pouches at 1,200 *g* for 10 min at 4°C, supernatants (20–100 μl) were incubated with N-tert-butoxy-carbonyl-L-alanine p-nitrophenyl ester (200 μM) for 10 min at 37°C and absorbances were determined at 414 nm in a Multiskan MCC 340 microplate reader (Labsystems, Helsinki, Finland) (Bustos *et al*., 1995).

Materials

[9,10-^{3}H]-oleic acid was purchased from Du Pont, (Itisa, Madrid, Spain); [5,6,8,11,12,14,15(n)-^{3}H]-PGE_2, [5,6,8,9,11,12,14,15(n)-^{3}H]-LTB_4 and L-[2,3,4,5-^{3}H]-arginine monohydrochloride were from Amersham Iberica (Madrid, Spain). iNOS polyclonal antiserum was purchased from Cayman Chem. (MI, U.S.A.) and NS398 from Universal Biologicals Ltd (London, U.K.). The rest of the reagents were from Sigma Chem. (MO, U.S.A.). *E. coli* strain CECT 101 was a gift from Prof. Uruburu, Department of Microbiology, University of Valencia, Spain, and antibody against LTB_4 was a gift from Dr S.J. Foster, Zeneca Pharmaceuticals, Macclesfield, Cheshire, U.K.

Statistical analysis

Data are expressed as mean ± s.e.mean. The results were analysed by Student's unpaired t test or by ANOVA followed by Dunnett's t test for multiple comparisons.

Results

Leukocyte infiltration and eicosanoid levels in the air pouch exudate

As shown in Figure 1, in saline-injected rats there was a low rate of cell accumulation in the air pouch exudate through the experiment. Total leukocyte counts ranged from $1.5 \pm 0.6 \times 10^6$ ml^{-1} at 2 h to $4.6 \pm 0.5 \times 10^6$ ml^{-1} at 24 h ($n = 6$). As in previous studies (Payá *et al*., 1996), we found that zymosan treatment induced a significant time-dependent increase in leukocyte infiltration in the pouch exudate that reached a peak by 8 h. Almost undetectable concentrations of PGE_2 or LTB_4 were found in saline-injected animals (Figure 2). In contrast, PGE_2 levels in exudates from zymosan-injected rats were maximal 4 h after zymosan injection and gradually declined with time, whereas the highest LTB_4 levels were observed at 2 h and almost disappeared by 6 h.

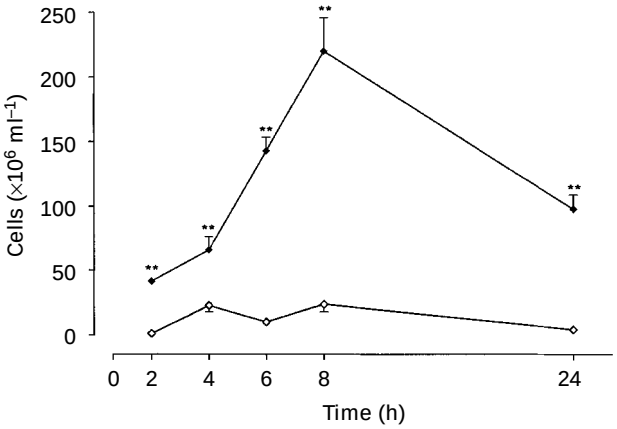

Figure 1 Time-course of the cellular infiltration in the rat air pouch. Each point is the mean for 6–12 animals; vertical lines show s.e.mean. The values observed for zymosan-injected rat air pouches (◆) were significantly different from saline-injected rat air pouches (◇) at $^{**}P < 0.01$ for all time points.

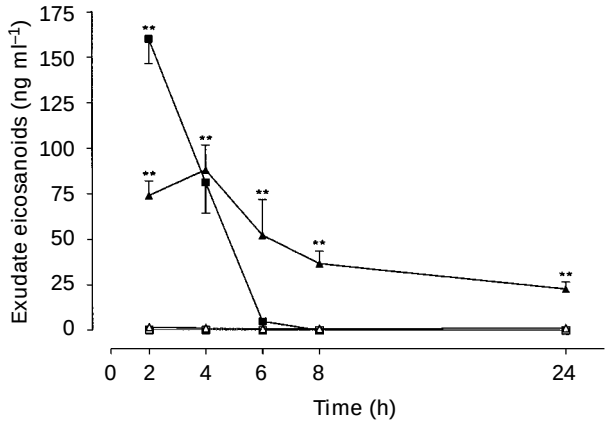

Figure 2 Time-course of prostaglandin E_2 (PGE_2) and leukotriene B_4 (LTB_4) accumulation in exudates of the rat air pouch. Results show the concentrations of PGE_2 (▲, △) and LTB_4 (■, □) in saline-injected rat air pouches (△, □) and zymosan-injected rat air pouches (▲, ■). Each point is the mean for 6–12 animals; vertical lines show s.e.mean. $^{**}P < 0.01$.

Accumulation of NO metabolites

Very low concentrations of NO metabolites were detected in saline-injected rats at each time point. Zymosan elicited a time-dependent increase in NO_2^- and $NO_2^- + NO_3^-$ which was measurable by 2 h and was maximal after 8 h (Figure 3).

Cellular NOS activity

Cytosolic fractions from the cell pellet obtained by centrifugation of pouch exudates were used to assay NOS activity by the citrulline method. As seen in Figure 4a, the cells from exudates of saline-treated animals did not show appreciable NOS activity, whereas injection of zymosan resulted in a marked NOS activity, significant even 2 h after zymosan (0.19 ± 0.07 nmol mg^{-1} citrulline detected in the zymosan group versus 0.002 ± 0.002 nmol mg^{-1} citrulline in the saline group, mean ± s.e.mean, $n = 6$, $P < 0.01$). This enzyme activity continued to increase during the first 8 h. When the cytosolic fraction from a pool of cells of 8 h zymosan-injected exudates was incubated with L-[^{3}H]-arginine + L-arginine, a time-dependent production of L-citrulline was observed (Figure 5). In another set of experiments we determined the influence of calcium and NADPH on this activity. After 30 min incuba-

tion, this enzyme generated 11.5 ± 0.4 nmol mg^{-1} protein of L-citrulline (mean $\pm$ s.e.mean, $n = 6$). This activity was not significantly affected when incubation was done in the presence of EGTA (100 μM), for 11.1 ± 0.3 nmol mg^{-1} protein of L-citrulline (mean $\pm$ s.e.mean, $n = 6$) was synthesized. Nevertheless in the absence of NADPH only 0.1 ± 0.1 nmol mg^{-1} protein of L-citrulline ($n = 6$) was detected.

Western blot analysis

Western blot analysis was carried out on cytosolic fractions of leukocytes obtained by centrifugation of exudates from air pouches with a polyclonal antiserum for iNOS. This antibody recognized iNOS from lungs of lipopolysaccharide-treated rats and failed to detect cNOS from rat brain (data not shown). The increase in NOS activity observed in cells of exudates from zymosan-injected animals was also reflected in an increase in the levels of a protein corresponding immunologically to the 130 kDa inducible isoform of NOS (Figure 4b). iNOS expression paralleled the time course of zymosan-induced NO_2^-/NO_3^- production and NOS activity, with a maximum at 8 h after zymosan. In contrast, iNOS immunoreactivity was not detected in cells of exudates from saline-injected animals. The level of iNOS protein in cytosolic fractions of cells from 8 h

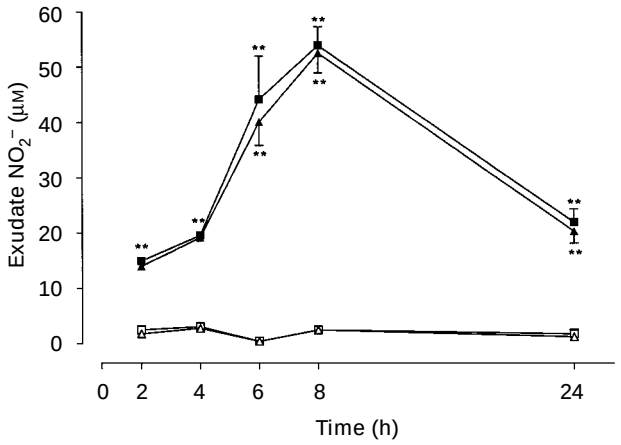

Figure 3 Time-course of the accumulation of NO metabolites in exudates of the rat air pouch. Results show the concentrations of NO_2^- (▲, △) and $NO_2^- + NO_3^-$ (expressed in NO_2^- equivalents) (■, □), in saline-injected rat air pouches (△, □) and zymosan-injected rat air pouches (▲, ■). Each point is the mean for 6–12 animals; vertical lines show s.e.mean. The values observed for zymosan-injected rat air pouches were significantly different from saline-injected rat air pouches at $^{**}P < 0.01$ for all time points.

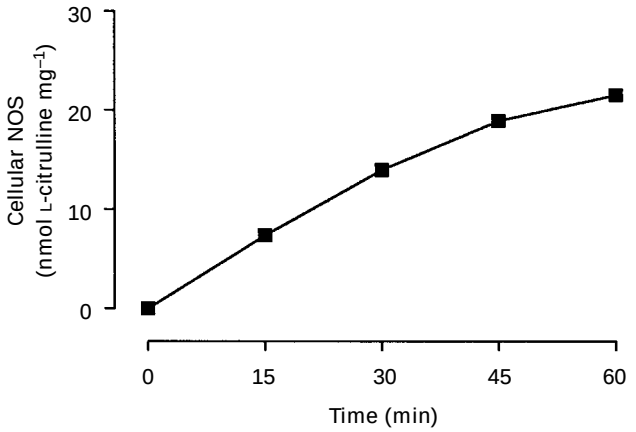

Figure 5 Time-dependence of NOS activity in cells present in exudates from 8 h zymosan-injected rat air pouches. Exudates from air pouches were collected 8 h after injection of zymosan and centrifuged as above. Pooled cytosolic fraction (40 μg protein) from cell pellets was used as the enzyme source and NOS activity was measured by the citrulline assay. Data are expressed as nmol of L-citrulline formed mg^{-1} of protein (means from $n = 6$). Standard errors (not shown) were less than 5% of the respective means.

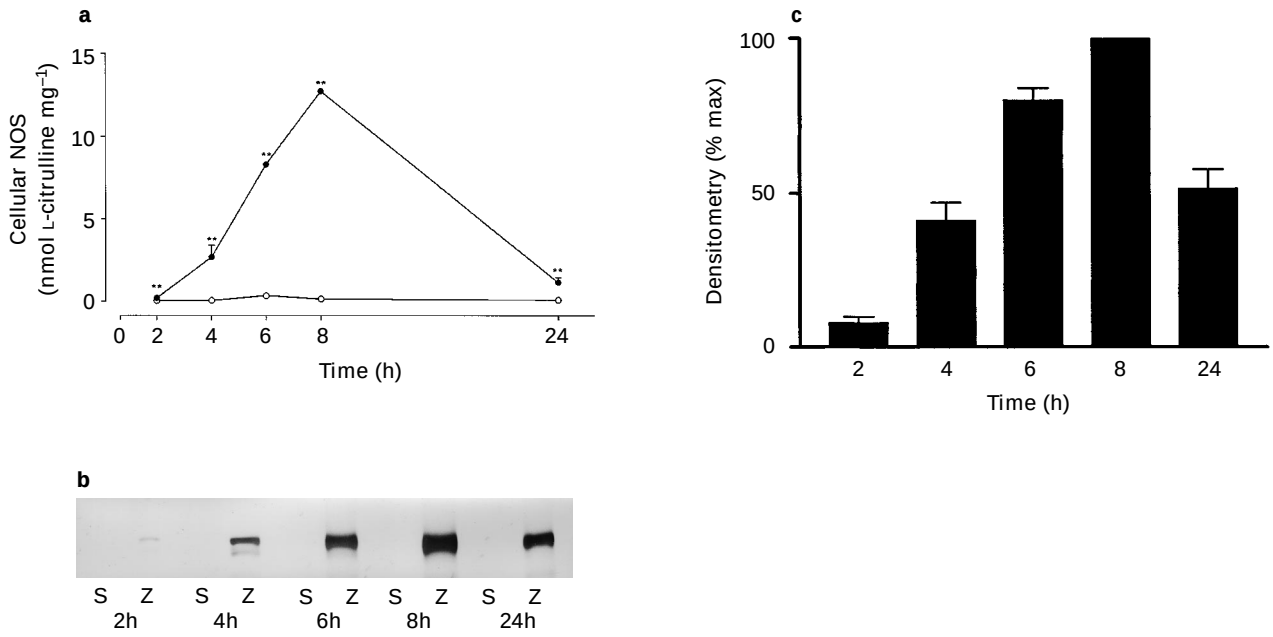

Figure 4 Time-course of iNOS changes in the cellular infiltrate of the rat air pouch. Exudates from air pouches were collected at different times and centrifuged. The cell pellet from each animal was homogenized separately. (a) NOS activity was measured in the cytosolic fraction by the citrulline assay, as described in the Methods section. Each point is the mean for 6–12 animals; vertical lines show s.e.mean. The values observed for zymosan-injected rat air pouches (●) were significantly different from saline-injected rat air pouches (○) at $^{**}P < 0.01$ for all time points. (b) iNOS protein levels were determined by Western blot. S = saline, Z = zymosan. The figure shows a representative blot from one of three experiments. (c) Densitometric analysis of blots for the groups treated with zymosan (mean $\pm$ s.e.mean, $n = 3$).

zymosan-injected rat air pouches in groups treated with the NOS inhibitors L-NAME or aminoguanidine was similar to that of 8 h zymosan control animals, while iNOS protein was attenuated with respect to the 8 h in zymosan control group when the animals were treated with dexamethasone (data not shown). The presence of maximal iNOS protein at 8 h after zymosan administration was confirmed by densitometry (Figure 4c).

NOS activity in different cell types

PMNs accounted for 92% of the infiltrating cells 8 h after zymosan, while only 5% monocytes and 3% lymphocytes were observed. This suggests that the high NOS activity detected in leukocytes present in exudates from 8 h zymosan-injected air pouches was due to PMNs. We then determined whether other cell types present in exudates also contained NOS activity. PMNs, monocytes and lymphocytes were separated as indicated in the Methods section and NOS activity was determined in their cytosolic fractions. This enzyme activity appeared in PMNs and monocytes, but not in lymphocytes, as is indicated by the generation values of 12.1 ± 0.8, 14.4 ± 2.0 and 0.0 ± 0.0 nmol mg^{-1} protein of L-citrulline, respectively (samples from 6 animals), that were observed.

Effect of colchicine

Colchicine was used to inhibit cellular infiltration in the zymosan-injected air pouch after 8 h, the time point of maximal NO production. Colchicine (1.5 mg kg^{-1}, i.p.), given 15 min before zymosan administration, inhibited cellular infiltration into the air pouch by 93% with respect to the zymosan group, with values lower than those observed in the saline group (Table 1); NO metabolites were inhibited by 99% and NOS activity by 93%.

Effects of enzyme inhibitors and dexamethasone

As maximal levels of iNOS activity, iNOS protein and NO metabolites were observed in exudates from 8 h zymosan-injected animals, this time point was chosen to assess the effects of NOS inhibitors and dexamethasone. As can be seen in Table 1, aminoguanidine and L-NAME did not modify cell influx into the air pouch, whereas dexamethasone exerted an inhibitory effect. However NO_2^- and $NO_2^- + NO_3^-$ levels as well as NOS activity were strongly reduced by NOS inhibitors and dexamethasone. Other parameters of this inflammatory reaction, like PGE_2 and secretory phospholipase A_2 activity ($sPLA_2$) (Payá *et al*., 1996) were also measured. The results of these experiments showed that only dexamethasone decreased PGE_2 levels in exudates (82% inhibition), while none of the drugs tested modified $sPLA_2$ activity. As shown in Table 2, leukocytes accumulating in exudates from air pouches injected with saline produced a low level of chemiluminescence, which was increased by TPA stimulation. The injection of zymosan stimulated the chemiluminescence of cells accumulating in exudates and this response was significantly inhibited in the groups treated *in vivo* with NOS inhibitors or dexamethasone. Another event of the inflammatory response to zymosan, the release of elastase activity by activated leukocytes, was slightly inhibited by administration of dexamethasone, whereas NOS inhibitors exhibited a lower inhibitory effect on elastase levels which was not significant if data were normalized in relation to the number of neutrophils present in pouch fluids (Table 2). Direct inhibitory effects of these drugs on elastase activity were discarded after *in vitro* incubations with supernatants of exudates from zymosan control groups (data not shown).

A peak of PGE_2 and low levels of NO metabolites, iNOS protein and NOS activity were observed 4 h after injection of zymosan. The effects of NOS inhibitors and dexamethasone were also determined at this time point of the zymosan re-

Table 1 Effects of NOS inhibitors and dexamethasone on the 8 h zymosan-injected air pouch

	Cells ($\times 10^6$ ml^{-1})	*NO_2^-* (μM)	*NO_2^-/NO_3^-* (μM)	*NOS* (nmol mg^{-1} citrulline)	*PGE_2* (ng ml^{-1})	*$sPLA_2$* (pmol ml^{-1} oleic acid)
Saline	23.9 ± 5.9**	1.3 ± 0.2**	1.8 ± 0.8**	0.1 ± 0.1**	0.1 ± 0.0**	35.1 ± 3.1**
Zymosan	218.9 ± 28.5	37.1 ± 4.0	39.8 ± 4.3	12.7 ± 0.7	36.9 ± 6.9	164.2 ± 7.8
Zymosan + L-NAME	227.4 ± 42.9	10.4 ± 1.6**	11.6 ± 4.9**	3.6 ± 0.3**	26.3 ± 5.3	170.7 ± 8.6
Zymosan + aminoguanidine	186.2 ± 10.0	11.4 ± 1.5**	12.1 ± 1.6**	3.7 ± 0.6**	48.1 ± 14.6	166.5 ± 5.2
Zymosan + dexamethasone	150.1 ± 9.7*	11.6 ± 1.4**	13.2 ± 2.2**	7.4 ± 0.7**	6.6 ± 2.3**	183.5 ± 5.6
Zymosan + colchicine	15.8 ± 4.3**	0.1 ± 0.1**	0.1 ± 0.1**	0.9 ± 0.3**	ND	ND

Results are the mean ± s.e.mean of $n = 6-12$ animals. *$P<0.05$; **$P<0.01$ ND-not determined. L-NAME and aminoguanidine (200 mg kg^{-1}, i.p.) were administered 30 min before zymosan injection and 4 h after zymosan the animals received an additional dose of 100 mg kg^{-1} by the same route. Dexamethasone (2 mg kg^{-1}, i.p.) was administered 1 h before zymosan and 4 h after the inflammatory stimulus the same dose was repeated. Colchicine (1.5 mg kg^{-1}, i.p.) was administered 15 min before the injection of zymosan.

Table 2 Effects of NOS inhibitors and dexamethasone on chemilumescence and elastase activity in the 8 h zymosan-injected air pouch

	Chemiluminescence (units/5×10^5 cells)		*Elastase* (nmol p-nitrophenol $10\ min^{-1}$)	(nmol p-nitrophenol $/10^6$ cells)
	−TPA	+TPA		
Saline	2409 ± 575**	29889 ± 6332	14.5 ± 3.3**	6.1 ± 1.6**
Zymosan	33618 ± 6873	53818 ± 8829	61.8 ± 1.5	14.0 ± 0.4
Zymosan + L-NAME	6001 ± 2820**	4213 ± 1445**	47.6 ± 4.8**	10.5 ± 1.1
Zymosan + aminoguanidine	16181 ± 1812*	24666 ± 1364**	42.0 ± 1.9**	11.2 ± 0.5
Zymosan + dexamethasone	5901 ± 1259**	8543 ± 1775**	27.8 ± 1.7**	9.7 ± 0.5*

Results are the mean ± s.e.mean of $n = 6-12$ animals. *$P<0.05$; **$P<0.01$ with respect to the zymosan control group. L-NAME and aminoguanidine (200 mg kg^{-1}, i.p.) were administered 30 min before zymosan injection and 4 h after zymosan the animals received an additional dose of 100 mg kg^{-1} by the same route. Dexamethasone (2 mg kg^{-1}, i.p.) was administered 1 h before zymosan and 4 h after the inflammatory stimulus the same dose was repeated. Chemiluminescence was measured for 4 min in the absence and presence of TPA.

sponse. In addition, we assessed the influence of the selective cyclo-oxygenase-2 inhibitor NS398 at this time of maximal prostaglandin production. As seen in Table 3, none of the compounds tested significantly affected either cell accumulation in exudates or $sPLA_2$ activity. As expected, the NOS inhibitors L-NAME and aminoguanidine decreased NO metabolites and NOS activity and had no effect on eicosanoids, including LTB_4, which was also determined because of the high levels present at this time (Payá *et al*., 1996). The glucocorticoid dexamethasone inhibited NO_2^- and $NO_2^- + NO_3^-$ levels and NOS activity, as well as eicosanoid levels. The increase in PGE_2 production after zymosan administration was inhibited by NS398. However, NO_2^-/NO_3^- levels and NOS activity were not affected by this COX-2 inhibitor.

Discussion

In the rat air pouch model of inflammation, our results indicate that zymosan injected into the air pouch induces NO formation, measured as NO_2^-/NO_3^-, with a peak at 8 h after zymosan administration. The accumulation of NO metabolites in exudates was dependent on migrating cells, as is demonstrated by the fact that colchicine pretreatment abolished NO_2^-/NO_3^-. Moreover, we detected NOS activity in the cellular component of exudates and there was a clear correlation between this activity and the time course of NO_2^-/NO_3^- levels in exudates. Release of NO (as assessed by measurements of NO_2^-/NO_3^-) and NOS activity were inhibited by the non-selective cNOS/iNOS inhibitor L-NAME and the more selective iNOS inhibitor aminoguanidine. The enzyme has the characteristics of iNOS, for it is calcium-independent and needs NADPH for activity. These observations indicate that iNOS accounts for NO release in the zymosan-injected rat air pouch. The induction of NOS by zymosan was confirmed by the detection of iNOS protein in high speed supernatants from the cellular fraction of exudates. It is interesting to note that the time course of the iNOS protein synthesis was found to coincide with the production of NO_2^-/NO_3^- and iNOS enzymatic activity.

The groups treated with dexamethasone showed an important decrease in NO_2^-/NO_3^- levels in exudates. In addition, the high speed supernatants of migrating leukocytes exhibited a decreased ability to convert L-arginine into L-citrulline, thus indicating an inhibition of iNOS expression by dexamethasone.

The iNOS activity present in migrating leukocytes was localized in PMNs and monocytes. Nevertheless, as the first cell type is the main component of cellular influx in these experimental conditions, the contribution of monocytes to the NOS activity observed in the cellular fraction of exudates can be minimal. Although the macrophage is an important source of NO in inflammation, induction of NOS activity by lipopolysaccharide has been shown in rat neutrophils (Kolls *et al*., 1994), as has its suppression by glucocorticoids, a process mediated in part by lipocortin 1 (Wu *et al*., 1995). Our results indicate the induction of NOS mainly in neutrophils and, therefore, NO derived from these cells may act as a mediator of their cytotoxic effects and participate in the inflammatory response.

It has been shown that zymosan induces an inflammatory response with activation of the complement alternative pathway (Schorlemmer *et al*., 1977; Wedmore & Williams, 1981; Jose, 1987) and release of inflammatory cytokines (Ferrándiz & Foster, 1991; Perretti *et al*., 1992). It is also known that cytokines induce NOS activity in macrophages and other cell types (Drapier *et al*., 1988; Knowles & Moncada, 1994), and therefore it is likely that cytokines mediate the NO production that we have observed in the inflammatory response induced by zymosan. On the other hand, there seem to be mutual interactions between the NOS and COX pathways. COX-2 activation by NO may lead to an exacerbated inflammatory response (Salvemini *et al*., 1993; 1995b), whereas the resulting increase in prostaglandin levels may in turn reduce the expression of iNOS (Sautebin *et al*., 1995). In fact, endogenous PGE_2 downregulates iNOS induction in rat mesangial cells (Tetsuka *et al*., 1995) and murine macrophages (Marotta *et al*., 1992). However, this interactive effect may depend on the concentration of NO for low levels of this mediator enhance COX activity (Salvemini *et al*., 1993; 1995b) but an increase in NO formation by iNOS could inhibit the expression of COX-2 and its enzymatic activity (Swierkosz *et al*., 1995).

In the rat air pouch model of inflammation, injection of zymosan has been shown to cause stimulation of arachidonic acid metabolism resulting in increased levels of PGE_2 and LTB_4 in exudates, with the peak of the first eicosanoid at 4 h and low levels at 8 h (Ferrándiz & Foster, 1991; Payá *et al*., 1996). Glucocorticoids are known to inhibit the induction of COX-2 (Fu *et al*., 1990; Masferrer *et al*., 1990; Masferrer *et al*., 1994) and NOS (Radomski *et al*., 1990; Salvemini *et al*., 1995a) without affecting constitutive enzymes. Our results suggest that COX-2 is the main enzyme responsible for the production of PGE_2 in this model, for dexamethasone strongly reduced the levels of this eicosanoid and the selective compound NS398 showed inhibitory effects. In our experiments, zymosan activated the COX and NOS systems with a different time frame: a peak for NO metabolites was observed at 8 h post zymosan injection, while PGE_2 production showed a maximum at 4 h. This suggests that there is an earlier phase in the zymosan air pouch inflammation, in which eicosanoids play a major role, and a later phase mediated by NO.

There may also be an interaction between the two pathways since the high levels of PGE_2 present at the initial times of this response may prevent iNOS induction and the amounts of NO metabolites, which increase with time, may progressively inhibit PGE_2 production. Therefore, it has been suggested that NOS inhibitors could modulate inflammatory responses by the dual inhibition of NO and prostaglandins (Salvemini *et al*., 1995a; 1996; Tracey *et al*., 1995). However, we have not obesrved modulatory actions of NO on COX activity. Our results indicate that systemic administration of NOS inhibitors

Table 3 Effects of different enzyme inhibitors and dexamethasone on the 4 h zymosan-injected air pouch

	Cells ($\times 10^6$ ml^{-1})	NO_2^- (μM)	NO_2^-/NO_3^- (μM)	*NOS* (nmol mg^{-1} citrulline)	PGE_2 (ng ml^{-1})	LTB_4 (ng ml^{-1})	$sPLA_2$ (pmol ml^{-1} oleic acid)
Saline	22.9±4.7**	2.8±0.4**	3.1±0.5**	0.05±0.05**	1.2±0.4**	0.2±0.1**	29.3±4.7
Zymosan	65.8±10.3	15.4±1.8	17.6±2.0**	4.0±0.6	88.2±13.7	81.3±16.9	28.6±5.2
Zymosan + L-NAME	52.2±3.1	2.0±0.5**	2.6±0.6**	1.9±0.2**	70.3±11.5	68.3±8.8	29.2±2.9
Zymosan + aminoguanidine	61.3±5.9	4.2±0.9**	5.2±1.0**	0.7±0.1**	79.8±16.8	66.1±11.1	24.1±2.5
Zymosan + dexamethasone	69.6±9.4	6.2±0.7**	6.8±0.7**	0.4±0.2**	17.1±1.5**	3.1±0.6**	27.5±5.1
Zymosan + NS398	63.8±12.5	20.2±1.9	21.8±2.0	3.1±0.7	29.3±4.3**	51.6±9.1	36.6±9.3

Results are the mean±s.e.mean of $n=6-12$ animals. **$P<0.01$. L-NAME (200 mg kg^{-1}, i.p.), aminoguanidine (200 mg kg^{-1}, i.p.) and NS398 (1 mg kg^{-1}, i.p.) were administered 30 min before zymosan injection. Dexamethasone (2 mg kg^{-1}, i.p.) was administered 1 h before zymosan.

to rats largely attenuated the production of NO metabolites in a specific inflammatory site caused by zymosan but they affected neither PGE_2 accumulation nor cellular influx in response to this inflammatory agent. This suggests that NO does not modulate these events. With respect to cell migration, the data obtained by Perretti *et al*. (1992) indicate the participation of interleukin-1 in rat pleuritis or mouse peritonitis induced by zymosan. Therefore, NOS inhibitors may not inhibit cytokine-mediated cell influx, which would be in agreement with previous observations on the migration induced by interleukin-1 administration in the mouse air pouch (Perretti *et al*., 1995). This is in contrast to the reduction in cell migration exerted by NOS inhibitors in different models (Tracey *et al*., 1995; Salvemini *et al*., 1996).

Interestingly, NOS inhibitors and dexamethasone administered *in vivo* inhibited chemiluminescence of leukocytes accumulating in air pouches at the time of maximal NO production and iNOS expression (8 h), although the higher reduction in chemiluminescence produced by L-NAME in comparison with aminoguanidine could be due in part to non-specific effects (Dikshit *et al*., 1996). In contrast, our results do not allow us to detect a clear interference of these drugs with the degranulation process.

On the other hand, the COX-2 inhibitor NS398 strongly inhibited PGE_2 levels but failed to modify NO_2^-/NO_3^- levels or NOS activity, thus showing that the endogenous release of prostaglandins does not affect NOS activity. In contrast to the inflammatory response induced by agents like carrageenan (Tomlinson *et al*., 1994; Salvemini *et al*., 1995a; 1996). COX-2 and iNOS are not co-induced in this experimental model.

Our results, therefore, do not support the hypothesis that endogenous NO participates in zymosan-stimulated prostaglandin biosynthesis and indicate that although NOS and COX-2 are induced by cytokines and other mediators in many inflammatory diseases, the possible modulation of COX by NO depends on the type of the inflammatory response involved. Finally, the induction of iNOS by phagocytic stimuli in migrating cells such as neutrophils results in the production of large quantities of NO, which may contribute to the pathophysiology of inflammatory disorders.

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