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ORIGINAL ARTICLE

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Co-regulation between cyclo-oxygenase-2 and inducible nitric oxide synthase expression in the time-course of murine inflammation

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Abstract Many in vitro studies have used cell cultures to focus on the relationships between cyclo-oxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) isoforms. We have investigated the time-course of regulation and the role of COX-2 and iNOS in a model of experimental inflammation in mice, the air pouch injected with zymosan. This study demonstrates that there is an early acute phase (4 h) mediated mainly by eicosanoids, with high levels of prostaglandin E_2 (PGE_2) produced by cyclo-oxygenase-1. In addition, in the later phase (from 12 h) there is a participation of nitric oxide (NO) and PGE_2 accompanied by co-induction of both iNOS and COX-2. These enzymes were detected in migrating leukocytes as well as in macrophages lining the air pouch. Administration of NS398 or indomethacin inhibited PGE_2 levels and COX activity, but also nitrite levels and iNOS activity, which was accompanied by a reduction in iNOS expression. Aminoguanidine inhibited nitrite levels and iNOS activity in addition to exerting inhibitory effects on the COX pathway. Treatment of animals with dexamethasone reduced nitrite and PGE_2 concentrations in air pouch exudates, as well as iNOS and COX-2 expression in migrating cells. Our results indicate that PGE_2 and NO may play in vivo mutual modulatory roles in the inflammatory response caused by zymosan injection into the mouse air pouch, a suitable model to study drugs acting on those pathways.

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Introduction

Nitric oxide (NO) is generated from L-arginine by nitric oxide synthase (NOS; for review see Knowles and Moncada 1994). Neuronal and endothelial NOS are constitutive, calcium-dependent isoforms, whereas the inducible, calcium-independent enzyme, inducible nitric oxide synthase (iNOS), has been found in macrophages, neutrophils and other cells activated by different stimuli. At present it is known that gene expression of constitutive enzymes may also be induced and that iNOS may function as a "constitutive" enzyme (for review see Michel and Feron 1997).

NO can have both anti-inflammatory and pro-inflammatory effects. This species plays an important role in host protection against some microorganisms, and its synthesis can retard inflammatory cell accumulation and activation in certain settings (for review, see Nathan 1997). On the other hand, NO mediates vasodilatation and facilitates leukocyte migration (Palmer et al. 1987; Wright et al. 1989; Sautebin et al. 1995). In addition, during inflammatory responses, increased levels of NO derived from iNOS activity would result in the formation of peroxynitrite after reaction with oxygen free radicals. This cytotoxic species is involved in vasodilatation and tissue damage (Demiryurek et al. 1998) mediated in part by poly(adenosine 5'-diphosphoribose) synthase activation (Szabo and Dawson 1998).

The participation of NO as a mediator of inflammation has been demonstrated in experimental models such as adjuvant arthritis (Ialenti et al. 1993; Stefanovic-Racic et al. 1994; Connor et al. 1995), arachidonic acid-induced paw oedema (Sautebin et al. 1995) and several responses to carrageenin in the rat (Iuvone et al. 1994; Tomlinson et al. 1994; Honoré et al. 1995; Salvemini et al. 1995b, 1996; Tracey et al. 1995), as well as in a model of chronic granulomatous inflammation in mice (Vane et al. 1994). It

has also been found that NO can be generated in the inflamed joints of patients with rheumatoid arthritis, leading to 3-nitrotyrosine detection in synovial fluid and blood serum (Kaur and Halliwell 1994).

Prostanoids regulate a number of physiopathological processes and are synthesized by two cyclo-oxygenase (COX) isoforms. COX-1 appears to synthesize the prostanoids involved in homeostatic functions, whereas COX-2, whose expression is restricted under basal conditions, is upregulated by inflammatory stimuli resulting in increased prostaglandin (PG) production (Ristimäki et al. 1994; Crofford 1997). However, there are controversial data (Wallace et al. 1998) in rats and mice which implicate COX-1 in the inflammatory responses. COX-2 expression in vivo has been reported in chronic inflammatory conditions such as rheumatoid arthritis (Kang et al. 1996) and in experimental models of inflammation (Masferrer et al. 1994; Tomlinson et al. 1994; Vane et al. 1994; Kang et al. 1996).

Many previous studies have used cell cultures to focus on the relationships between NOS and COX metabolites. However, little is known about the mechanisms regulating the two pathways in inflammatory responses in vivo. Co-induction of iNOS and COX-2 has been demonstrated in lipopolysaccharide (LPS)-treated rats (Salvemini et al. 1995a), rat carrageenin-induced pleurisy (Tomlinson et al. 1994), rat air pouch injected with carrageenin (Salvemini et al. 1995b) and murine air pouch granulomatous inflammation (Vane et al. 1994). Zymosan is a phagocytic stimulus which induces an inflammatory reaction characterized by the activation of the alternative pathway of complement and C5a formation (Konno and Tsurufuji 1983), accompanied by synthesis of eicosanoids and cytokines (Ferrándiz and Foster 1991). Nevertheless, there are differences in the inflammatory response to zymosan depending on the experimental setting. In mice, intraperitoneal injection of zymosan determines a marked increased in COX and 5-lipoxygenase products, with a minimal contribution on the part of complement fragments (Rao et al. 1994). In contrast, NO but not PGs, would mediate zymosan-induced plasma extravasation in rat skin (Ridger et al. 1997) and we have recently reported the independent participation of iNOS and COX metabolites in the zymosan-injected rat air pouch model of inflammation (Payá et al. 1997).

We have investigated the roles of iNOS and COX-2 in a model of experimental inflammation in mice, the air pouch injected with zymosan, an in vivo assay widely used to evaluate inhibitors of eicosanoid production. Our results show that NO and PGs play mutual modulatory roles.

Materials and methods

Mouse air pouch. Air pouches were produced by subcutaneous injection of 10 ml sterile air into the back of female Swiss mice (25–30 g). Three days later 5 ml sterile air was injected into the same cavity (Edwards et al. 1981). Six days after the initial air injection, 1 ml sterile saline or 1 ml 1% w/v zymosan in saline was injected into the air pouch. In the 4 h zymosan-injected air pouch, aminoguanidine (200 mg/kg), indomethacin (10 mg/kg) or NS398 (10 mg/kg) was administered i.p. 30 min before the zymosan injection. Dexamethasone (2 mg/kg) was administered i.p. 1 h before zymosan. In the 12 h zymosan-injected air pouch, two doses of aminoguanidine (200 mg/kg i.p.) were administered 6 h and 9 h after zymosan injection. Dexamethasone (2 mg/kg i.p.) was administered 1 h before zymosan, and 8 h after the inflammatory stimulus the same dose was repeated. In another set of experiments, animals received colchicine (1.5 mg/kg i.p.) 30 min before the injection of zymosan, and 8 h after the stimulus an additional dose was injected. Indomethacin and NS398 were administered at 10 mg/kg i.p. 30 min prior to zymosan, and 8 h after the stimulus the same dose was repeated in the NS398 group. At different time intervals after the injection of saline or zymosan into the air pouch, the mice were killed by cervical dislocation and the exudate in the pouch was collected with 1 ml of saline. Leukocytes present in exudates were measured using a Coulter counter and differential counting was also performed. After centrifugation of exudates at 1200 *g* for 10 min at 40°C, the supernatants were used to measure LTB_4 and PGE_2 levels by radioimmunoassay (RIA) (Moroney et al. 1988), and nitrite levels according to the method described by Misko et al. (1993). The cell pellet was used to measure COX and NOS activities and for western blotting. Protein was quantified by the Bradford technique (Bradford 1976) using BSA as standard.

COX assay. Cell pellets obtained by centrifugation of exudates at 1200 *g* for 10 min at 40°C were resuspended in 10 mM HEPES pH 7.4 containing saccharose (0.32 M), EDTA (100 μM), dithiothreitol (1 mM), phenylmethylsulfonyl fluoride (1 mg/ml) and leupeptin (10 μg/ml) (Knowles et al. 1990) and sonicated (3×10 s) at 40°C in an ultrasonicator at maximum potency. The resulting homogenate was centrifuged at 1200 *g* for 10 min at 40°C, followed by centrifugation of the supernatant at 100,000 *g* for 100 min at 40°C. The cytosolic fractions were used for the assay of NOS. Microsomes (20 μg protein) were incubated for 30 min at 37°C in 50 mM TRIS-HCl, pH 7.4, with 5 μM arachidonic acid in the presence of 2 μM hematin and 1 mM L-tryptophan. The reaction was terminated by boiling the samples for 5 min, and PGE_2 levels were determined by RIA (Moroney et al. 1988).

NOS assay. NOS activity was determined in high speed supernatants by monitoring the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline (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). 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 1:1 suspension of Dowex (50 W) in water. Radioactivity was measured in supernatants by liquid scintillation counting.

Western blot analysis. High speed supernatants and microsomal fractions from leukocytes present in pouch exudates were obtained as indicated for COX activity. For iNOS, 25 μg protein (or 35 μg in the study of enzyme inhibitors) were loaded on 12% PAGE-SDS and transferred onto nitrocellulose membranes for 90 min at 125 mA. Membranes were blocked in PBS-Tween 20 containing 3% w/v BSA and incubated with anti-iNOS polyclonal antibody (1/1000 dilution; Cayman Chemical, USA). For iNOS detection, blots were washed and incubated with peroxidase-conjugated goat anti-rabbit IgG (1/20,000 dilution; Dako, Denmark). For COX-1 and COX-2, equal aliquots of microsomal fraction protein (25 μg, or 35 μg in the study of enzyme inhibitors) were electrophoresed on 12.5% PAGE-SDS and proteins were transferred onto nitrocellulose membranes, which after blocking were incubated with COX-1 monoclonal antiserum (1/1000 dilution; Cayman Chemical) or COX-2 polyclonal antiserum (1/1000 dilution; Santa Cruz, USA), respectively, followed by incubation with the respective secondary antibodies: horseradish peroxidase-conjugated goat anti-mouse IgG (1/20,000 dilution; Sigma, USA) or monoclonal

anti-goat/sheep peroxidase-conjugated (1/20,000 dilution; Sigma). The immunoreactive bands were visualized using an enhanced chemiluminescence system (ECL; Amersham, USA).

Cells. Leukocytes present in exudates from 12 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 monocyte/lymphocyte population was resuspended in RPMI-1640 media pH 7.4 with 10% fetal bovine serum and 2 mM L-glutamine and was incubated at a cell density of 10^7/ml 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 cells (90% monocytes) were collected. The monocyte and neutrophil fractions were sonicated and treated for western blotting as indicated above.

Immunofluorescence analysis. Pouch tissues were collected from animals belonging to the 24-h zymosan-injected group and the corresponding saline group and fixed in 10% formalin. Pouch lining cells were detached on glass slides treated with poly-L-lysine. Cells were examined microscopically under bright-field or with epifluorescence. iNOS or COX-2 was identified by the corresponding specific rabbit polyclonal antibody (1/100 dilution, 2 h, 37°C), followed by incubation with a TRITC-conjugated goat anti-rabbit IgG (1/1000 dilution; Sigma). To identify the monocyte/macrophage population, the tissue was incubated with rat anti-mouse macrophage-FITC (1/100 dilution, Oxford, UK).

Materials. [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 purchased from Amersham Iberica (Spain). Inhibitor NS398 was from Cayman Chemical. The rest of the reagents were from Sigma. The antibody against LTB_4 was a gift from Dr. S.J. Foster, Zeneca Pharmaceuticals, Macclesfield, Cheshire, UK.

Results

Time-course of production of eicosanoids, cellular accumulation, COX activity and protein expression

In 6-day-old mouse air pouches, concentrations of eicosanoids in pouch exudates were almost undetectable in saline-injected animals (Fig. 1A,B), but after zymosan injection, there was a rapid generation of eicosanoids, with maximum levels of LTB_4 and PGE_2 at 4 h. The exudate content of the first eicosanoid sharply declined afterwards and almost disappeared at 12 h, whereas PGE_2, after peaking, showed sustained levels between 8 and 24 h followed by a slow decrease.

The peaks in eicosanoid levels preceded the maximal cell migration into the air pouch. As shown in Fig. 1C, zymosan treatment induced a significant increase in leukocyte counts in the pouch exudate that exhibited the highest values at 8–12 h and declined with time. The migrating leukocytes were predominantly neutrophils (92, 88 and 89% at 4, 12 and 24 h, respectively), with some lymphocytes (6–8%) and monocytes (1, 4 and 5% at the same times, respectively).

As shown in Fig. 2A, COX activity assayed in microsomal fractions of cells migrating into the pouch exudate showed a significant value at 4 h followed by slow in-

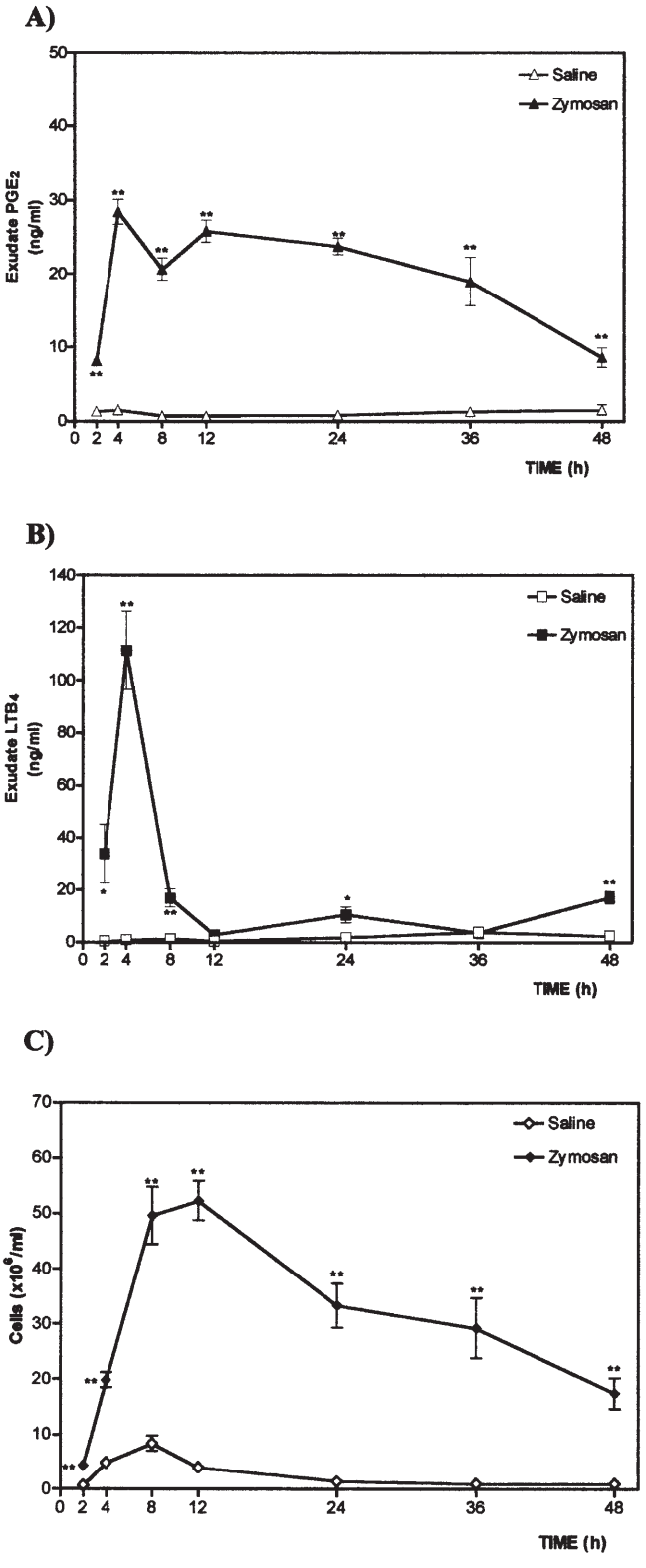

Fig. 1 A–C Time-course of eicosanoid levels and cellular infiltration in exudates of the mouse air pouch. **A** Concentrations of PGE_2. **B** Concentrations of LTB_4. **C** Total cell counts. Each point is the mean ± SEM (n=6–12 animals). *Closed symbols* represent the values observed for zymosan-injected air pouches and *open symbols* for saline-injected air pouches. *P<0.05; **P<0.01

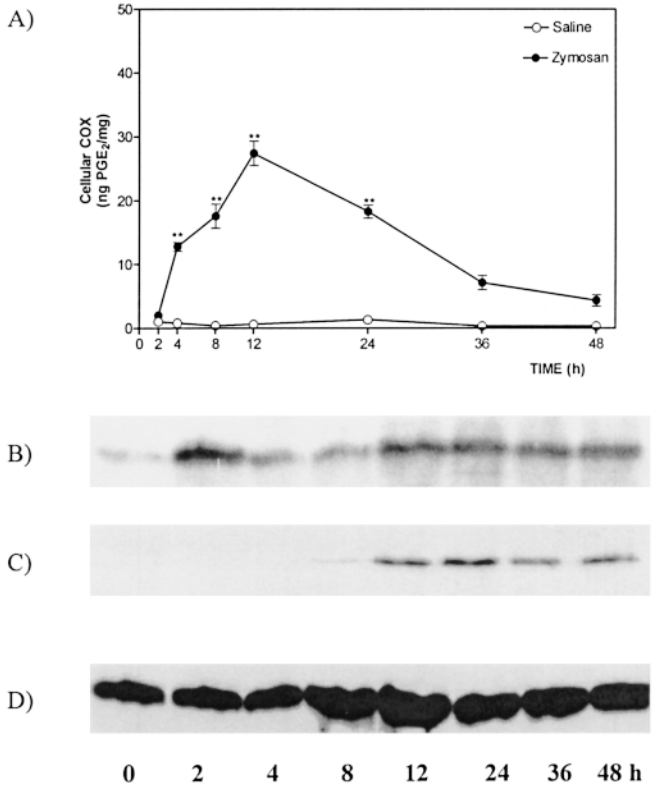

Fig. 2 A–D Time-course of COX activity and expression in cells from exudates of the mouse air pouch. **A** COX activity. **B** COX-1 expression. **C** COX-2 expression. **D** β-Actin expression. COX activity was measured in the microsomal fraction of exudated cells; each point is the mean ± SEM (*n*=6–12 animals). *Closed symbols* represent the values observed for zymosan-injected air pouches and *open symbols* for saline-injected air pouches. **P*<0.05; ***P*<0.01. Western blot analysis: COX-1 and COX-2 proteins were not detected in cells from saline-injected air pouches. The figure shows the time course of COX-1 (**B**), COX-2 (**C**) and β-actin (a house-keeping reference protein; **D**) protein expression in cells from zymosan-injected air pouches and it is representative of three similar experiments

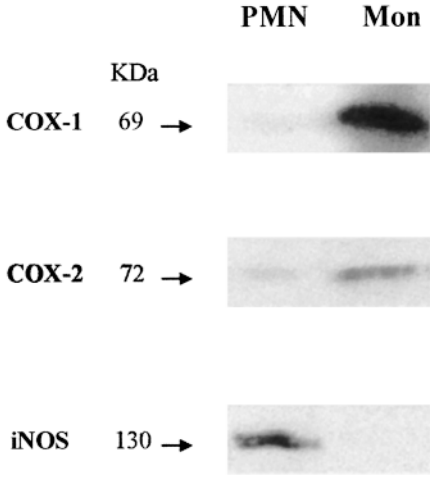

Fig. 3 Western blots demonstrating COX-1, COX-2 and iNOS expression in neutrophils (*PMN*) and monocytes (*Mon*) from exudates of zymosan-injected (12 h) mouse air pouch. The figure is representative of three similar experiments

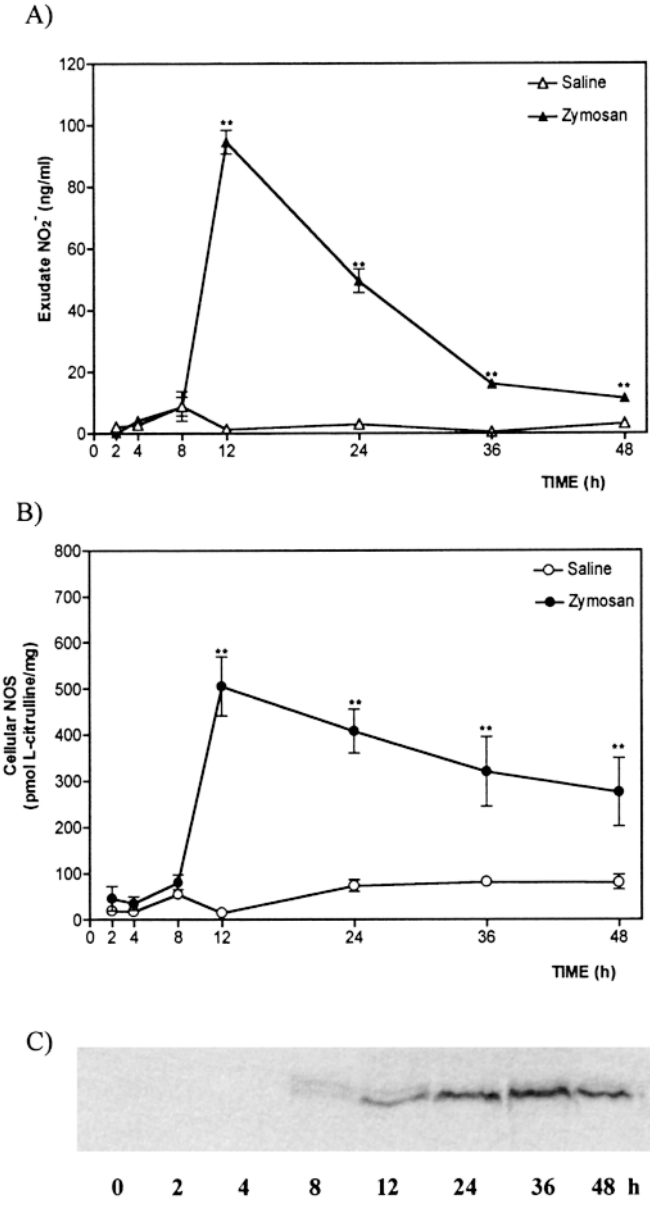

Fig. 4 A–C Time-course of nitrite levels in exudates and iNOS activity and expression in cells from exudates of the mouse air pouch. **A** Nitrite concentration. **B** iNOS activity. **C** iNOS expression. For nitrite levels and iNOS activity, each point is the mean ± SEM (*n*=6–12 animals). *Closed symbols* represent the values observed for zymosan-injected air pouches and *open symbols* for saline-injected air pouches. ***P*<0.01. Western blot analysis: iNOS protein was not detected in cells from saline-injected air pouches. **C** shows the time course of iNOS protein expression in cells from zymosan-injected air pouches and is representative of three similar experiments

creases with time up to 12 h. This activity then decreased dramatically. Western blot analysis for COX-1 and COX-2 was carried out on microsomal fractions of leukocytes obtained by centrifugation of air pouch exudates. Low levels of COX-1 were detected in saline-injected mice, while COX-2 was absent (data not shown). In zymosan-injected animals, after 2 h there was an increase in the levels of a

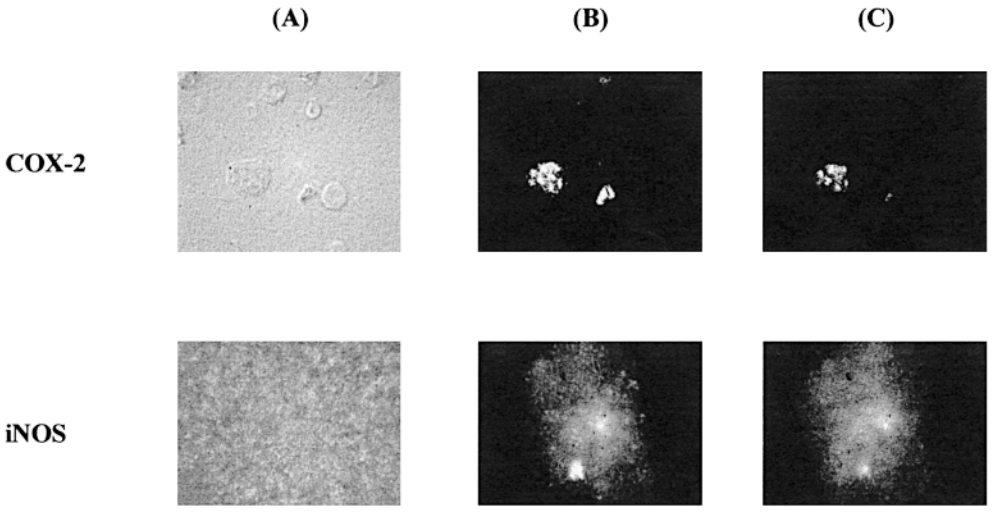

Fig. 5 A–C Immunofluorescence analysis of COX-2 and iNOS in cells lining the mouse air pouch injected with zymosan (24 h). Cells were examined microscopically under bright-field (**A**) or with epifluorescence (**B,C**). COX-2 or iNOS of cells was identified by staining with rabbit polyclonal antibody to COX-2 or iNOS. TRITC-conjugated goat anti-rabbit IgG was used to react with tissue bound anti-COX-2 or anti-iNOS antibodies (**B**). To identify the nocytes/macrophages the tissue was stained with a monocyte/macrophage specific intracellular antigen of the mouse (rat anti-mouse macrophage-FITC; **C**)

protein corresponding immunologically to COX-1, without significant variations in this protein from 12 h (Fig. 2B), whereas COX-2 protein was not detected until 8 h after zymosan administration and its expression increased afterwards (Fig. 2C). Western blot analysis for COX-1 and COX-2 in the monocyte/neutrophil population present in exudates from 12 h was positive for COX-1 and COX-2 in monocytes and to a lesser extent for COX-2 in neutrophils (Fig. 3).

Time-course of production of nitrite, iNOS activity and protein expression

Very low concentrations of nitrite were detected in saline-injected mice at every time point (Fig. 4A). In contrast, a sharp increase in nitrite levels was observed in pouch exudates 12 h after zymosan, coinciding with the maximum NOS activity assayed in cytosolic fractions from the cell pellet obtained by centrifugation of pouch exudates (Fig. 4B). The cells from exudates of saline-treated animals did not show appreciable NOS activity (data not shown).

Western blot analysis for iNOS was carried out on cytosolic fractions of leukocytes present in air pouch exudates. In zymosan-injected animals there was an increase in the levels of a protein corresponding immunologically to the 130 kDa inducible isoform of NOS (Fig. 4C) which was not detected in saline-injected animals. iNOS expression was detected at 8 h after zymosan with a maximum at 24–36 h. Western blot analysis for iNOS in the monocyte/neutrophil population cells present in exudates from 12 h was positive only for neutrophils (Fig. 3).

Immunofluorescence analysis

The air pouch cavity is lined with monocyte/macrophage cells which could contribute to the production of inflammatory mediators. iNOS and COX-2 were identified in cells previously detached with poly-L-lysine, by the corresponding primary specific rabbit polyclonal antibodies followed by reaction with a TRITC-conjugated anti-rabbit antibody. In parallel, the monocyte/macrophage population was identified with a specific anti-mouse macrophage-FITC antibody. The immunofluorescence analysis of those cells lining the air pouch (24 h after zymosan) indicated the presence of COX-2 and iNOS in the monocyte/macrophage population (Fig. 5).

Effect of colchicine

We determined the effect of colchicine at the time of maximal nitrite production and iNOS and COX activities (12 h after zymosan). Colchicine partially reduced cellular infiltration into the air pouch in our experimental conditions. The group treated with colchicine (1.5 mg/kg i.p.) showed reduced total leukocyte counts in exudates ($28.0\pm3.6\times10^6$/ml) with respect to the control group ($52.5\pm3.6\times10^6$/ml, n=6, P<0.01). Interestingly, this inhibition of cellular influx was accompanied by a similar re-

Table 1 Effects of enzyme inhibitors and dexamethasone on the 4-h zymosan-injected air pouch. Indomethacin (10 mg/kg), NS398 (10 mg/kg) and aminoguanidine (200 mg/kg) were administered i.p. 30 min prior to zymosan. Dexamethasone (2 mg/kg) was administered i.p. 1 h prior to zymosan. Results are the mean ± SEM of n=6–12 animals

	Cells ($\times10^6$/ml)	LTB_4 (ng/ml)	PGE_2 (ng/ml)	COX (ng PGE_2/mg)
Saline	2.4±0.7*	1.0±0.3*	1.6±0.4*	0.5±0.2*
Zymosan	19.5±1.5	104.8±7.9	27.5±2.3	11.7±0.8
Zymosan+ indomethacin	15.9±2.1	110.0±7.0	1.1±0.1*	6.5±0.7*
Zymosan+NS398	22.3±2.9	104.6±6.2	22.5±4.0	13.6±0.9
Zymosan+ aminoguanidine	20.3±1.8	79.6±8.9	25.3±1.5	9.2±1.4
Zymosan+ dexamethasone	25.8±2.5	51.2±9.4*	13.4±1.5*	14.8±3.2

*P<0.01 compared with the zymosan group

Table 2 Effects of enzyme inhibitors and dexamethasone on the 12 h zymosan-injected air pouch. Indomethacin and NS398 were administered at 10 mg/kg i.p. 30 min prior to zymosan, and 8 h after the stimulus the same dose was repeated in the NS398 group. Aminoguanidine (200 mg/kg, i.p.) was administered 6 h and 9 h after zymosan injection. Dexamethasone (2 mg/kg i.p.) was administered 1 h prior to zymosan, and 8 h after the inflammatory stimulus the same dose was repeated. Results are the mean ± SEM of n=6–12 animals

	Cells ($\times 10^6$/ml)	Nitrite (ng/ml)	NOS (pmol citrulline/mg)	PGE_2 (ng/ml)	COX (ng PGE_2/mg)
Saline	4.8±1.3**	6.6±2.7**	0.0± 0.0**	0.7±0.1**	0.3±0.1**
Zymosan	50.6±2.3	94.7±4.8	504.3±64.0	23.3±1.8	26.7±2.2
Zymosan+indomethacin	35.8±2.2*	60.8±4.8**	187.8±42.9**	0.3±0.1**	13.4±3.0**
Zymosan+NS398	55.0±6.6	50.7±4.7**	80.3±21.6**	8.4±2.2**	11.7±1.6**
Zymosan+aminoguanidine	39.1±5.2	0.0±0.0**	79.6± 6.1**	15.5±0.9**	16.6±2.5**
Zymosan+dexamethasone	41.4±2.6	65.4±5.8**	177.9±63.5**	4.8±1.9**	16.5±2.0**

*P<0.05 compared with the zymosan group
**P<0.01 compared with the zymosan group

duction in nitrite concentration (60.8±10.5 versus 106.0±9.8 ng/ml, n=6, P<0.05) and PGE_2 levels (9.8±1.1 versus 24.3±2.2 ng/ml, n=6, P<0.01). In these cells a partial decrease in NOS activity was also observed (245.2±70.0 versus 562.8±75.0 pmol citrulline/mg protein, n=6, P<0.05) accompanied by a weak modification of COX activity (19.3±2.5 versus 29.5±2.5, n=6, P<0.05).

Effects of enzyme inhibitors and dexamethasone

We assessed the in vivo effects of COX and NOS inhibitors and dexamethasone at the time points of maximal levels of PGE_2 (4 h), COX activity, nitrite and iNOS activity (12 h). As can be seen in Table 1, 4 h after zymosan none of the compounds tested significantly modified cell influx into the air pouch. PGE_2 and COX activity were inhibited in the animals treated with indomethacin, whereas aminoguanidine and NS398 failed to significantly modify any parameter. Dexamethasone was the only compound able to decrease LTB_4 and PGE_2 levels, which would suggest a possible inhibition of phospholipase A_2 resulting in a decreased substrate availability. Twelve hours after zymosan injection, nitrite levels and COX and iNOS activities showed a peak that coincided with high levels of PGE_2. At this time point, cell migration was slightly inhibited by indomethacin (Table 2). Dexamethasone, indomethacin, NS398 and aminoguanidine significantly reduced nitrite and PGE_2 concentrations, as well as iNOS and COX activities. As expected, direct inhibitory effects of NS398 or indomethacin on iNOS activity, and of aminoguanidine on COX activity, were ruled out after in vitro incubations of enzyme preparations from the control group with a concentration of 0.1 mM of the corresponding drug (data not shown). The effect of these drugs on COX-2 and iNOS protein expression is shown in Fig. 6. Dexamethasone reduced the expression of both proteins, in contrast with aminoguanidine which did not exert any significant effect. COX inhibitors showed a weak effect on COX-2 expression, whereas indomethacin was more effective than NS398 in inhibiting iNOS expression.

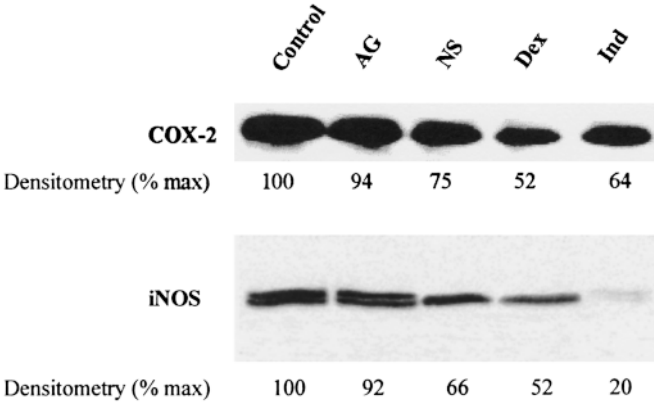

Fig. 6 Effect of enzyme inhibitors on COX-2 and iNOS expression and densitometric analysis in cells from exudates of zymosan-injected (12 h) mouse air pouch. The figure is representative of three similar experiments. *AG* Aminoguanidine, *NS* NS398, *Dex* dexamethasone, *Ind* indomethacin

Discussion

The early phase of the zymosan-injected mouse air pouch (4 h) is mediated by eicosanoids, whereas NO does not play a role. The fact that indomethacin inhibited PGE_2 accumulation in the exudate while NS398 did not, suggests that COX-1 was the enzyme responsible for PG synthesis at that time. This is in agreement with the increase in COX activity observed after zymosan administration, in the cells migrating into the air pouch and maximal PGE_2 levels in exudates. Nevertheless, at 2 h there was a discrepancy between COX-1 expression and COX activity, which could be due to the lack in the cell exudate of some cofactor required for activity (Capdevila et al. 1995).

In contrast, our results show the participation of NO and PGE_2 in the later phase of this experimental model, from 12 h after the administration of the inflammatory stimulus. Zymosan causes the co-induction of both iNOS and COX-2 in the mouse air pouch, with resulting increases in NO (measured as nitrite) and PGE_2. A high level of these enzyme activities was detected in migrating leukocytes, accompanied by COX-1, COX-2 and iNOS expression in the neutrophil/monocyte population cells. In

this regard, neutrophils expressed iNOS and to a lesser extent COX-2, whereas monocytes showed a higher level of COX-1 expression as compared with COX-2.

Colchicine treatment partially reduced leukocyte influx and nitrite and PGE_2 levels, thus indicating that migrating leukocytes play a role in the production of these metabolites. Nevertheless, iNOS and COX-2 expression was also observed after zymosan treatment in the macrophages lining the air pouch, which suggests that they may also contribute to nitrite and PGE_2 levels in exudates.

Although at this later phase COX-1 is still present, COX-2 plays an important role in PG synthesis, evidenced by the fact that NS398 inhibited PGE_2 levels and COX activity. As expected, the selective iNOS inhibitor aminoguanidine inhibited nitrite levels and iNOS activity but did not significantly modify cellular migration into the exudates. This is in agreement with the reported inability of NOS inhibitors to affect cell influx induced by interleukin-1 in the mouse air pouch (Perretti et al. 1995) since it is known that this cytokine participates in zymosan-induced inflammatory responses in mice (Perretti et al. 1992). The results obtained with dexamethasone lend support to the idea that zymosan induces these inflammatory enzymes in vivo, since glucocorticoids inhibit the expression of COX-2 (Fu et al. 1990; Masferrer et al. 1990, 1994) and iNOS (Radomski et al. 1990; Salvemini et al. 1995b).

The interactions between the iNOS and COX pathways can lead to controversial results depending on the cell system and experimental conditions. In some systems, both pathways are induced by inflammatory stimuli but they seem to act in an independent manner, as observed in rat aorta in culture stimulated with LPS (Bishop-Bailey et al. 1997) or in the response to zymosan in the rat air pouch (Payá et al. 1997).

On the contrary, the possibility exists of an interaction between NO and COX. In vitro, it has been reported that NO attenuates the synthesis of PGE_2 in chondrocytes from osteoarthritic patients (Amin et al. 1997), and it has been suggested that NO is involved in the negative regulation of COX-2 expression in rat peritoneal macrophages (Habib et al. 1997) and J774.2 macrophages (Swierkosz et al. 1995). Nevertheless, there are reports that NO potentiates cytokine-induced PGE_2 production in a number of cell systems such as vascular smooth muscle cells (Inoue et al. 1993), rat mesangial cells (Tetsuka et al. 1994), human microglial cells (Janabi et al. 1996) and human airway epithelial cells (Watkins et al. 1997). This effect of NO could be due to an amplification of COX-2 expression (Tetsuka et al. 1996). However, in a murine model of air pouch granulomatous inflammation, it is suggested that NO produced by iNOS seemed to inhibit the induction of COX-2, and low levels of NO appeared to activate COX (Vane et al. 1994). A regulatory role of NO in the in vivo production of prostanoids has been observed in rats treated with LPS (Salvemini et al. 1995a), in the carrageenin rat air pouch (Salvemini et al. 1995b) and in a model of renal inflammation in rabbits, where NO released from iNOS could activate the induced COX, resulting in increased production of PGs (Salvemini et al. 1994). Our results are in agreement with these previous reports in other species since the administration of aminoguanidine largely reduced COX activity and the content of PGE_2 in exudate, without affecting the expression of COX-2. As a consequence, inhibitors of iNOS may reduce inflammation by the dual inhibition of NO and PGs (Salvemini et al. 1995a,b).

On the other hand, COX metabolites could affect NOS activity. Some studies have shown that cAMP and PGE_2 can enhance COX-2 expression but not iNOS in rat mesangial cells stimulated by cytokines or LPS (Nüsing et al. 1996), and a dual behaviour is also plausible, since small increases in cAMP may enhance NO synthesis in ANA-1 macrophages while larger increases may inhibit it (Mullet et al. 1997). PGE_2 could inhibit nitrite production in J774.2 cells stimulated with LPS (Marotta et al. 1992) and downregulates iNOS induction in rat mesangial cells (Tetsuka et al. 1994). In contrast, inhibition of COX activity by indomethacin results in a reduction in nitrite accumulation in rat Kupffer cells (Gaillard et al. 1992), as well as in rat alveolar macrophages, where it has been demonstrated an inhibitory effect of indomethacin and other non-steroidal anti-inflammatory drugs on iNOS activity and gene expression (Aeberhad et al. 1995). In vivo, it has been reported a slight inhibition of nitrite production by NS398 (Salvemini et al. 1995b). In our experiments, when animals were treated with a selective inhibitor of COX-2, NS398, or the dual inhibitor of COX-1 and COX-2 indomethacin, lower levels of iNOS activity and nitrite present in exudates were observed. In addition, western blot analysis indicated an inhibitory effect of indomethacin on the expression of COX-2 and mainly on iNOS, in the leukocytes migrating into the air pouch exudate. Our results would indicate a correlation between the reduction in PGE_2 levels in exudates and the inhibition of iNOS expression in the migrating cells, thus suggesting that prostanoids could regulate iNOS expression and NO production during the inflammatory response in vivo.

In summary, this work offers further insight into the relationships between iNOS and COX pathways in the inflammatory response in vivo. Our data indicate that NO derived from iNOS expressed in migrating leukocytes and in macrophages lining the air pouch contributes to zymosan-induced inflammatory response in the mouse air pouch, 12–48 h after the injection of zymosan, simultaneously with the induction of COX-2. PGE_2 could be produced mainly by COX-1 activity during the early phase, peaking at 4 h, and from 12 h, COX-2 could be induced in the migrating cells and the macrophages lining the air pouch, leading to persistent levels of PGE_2 in the exudate. Our experiments show that PGs released by zymosan-induced inflammation may be regulated via NO, which would facilitate the activity of COX. The levels of NO may, in turn, be influenced by PGs, which would upregulate iNOS. Thus, PGs and NO may play mutual modulatory roles in this experimental model of inflammation, which is suitable for evaluating drugs affecting those pathways.

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