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Inducible isoforms of cyclooxygenase and nitric-oxide synthase in inflammation

(cytokines/prostanoids/granuloma)

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ABSTRACT **Cyclooxygenase (COX) converts arachidonic acid to prostaglandin H_2, which is further metabolized to prostanoids. Two isoforms of COX exist: a constitutive (COX-1) and an inducible (COX-2) enzyme. Nitric oxide is derived from L-arginine by isoforms of nitric-oxide synthase (NOS; EC 1.14.13.39): constitutive (cNOS; calcium-dependent) and inducible (iNOS; calcium-independent). Here we have investigated inducible isoforms of COX and NOS in the acute, chronic, and resolving stages of a murine air pouch model of granulomatous inflammation. COX and NOS activities were measured in skin samples in the acute phase, up to 24 h. Activities in granulomatous tissue were measured at 3, 5, 7, 14, and 21 days for the chronic and resolving stages of inflammation. COX-1 and COX-2 proteins were assessed by Western blot. COX activity in the skin increased over the first 24 h and continued to rise up to day 14. COX-2 protein rose progressively, also peaking at day 14. COX-1 protein remained unaltered throughout. The iNOS activity increased over the first 24 h in the skin, with a further major increase in the granulomatous tissue between days 3 and 7, followed by a decrease at day 14 and a further increase at day 21. The rise in COX and NOS activities in the skin during the acute phase reinforces the proinflammatory role for prostanoids and suggests one also for nitric oxide. However, in the chronic and resolving stages, a dissociation of COX and NOS activity occurred. Thus, there may be differential regulation of these enzymes, perhaps due to the changing pattern of cytokines during the inflammatory response.**

Inflammation is a complex process involving numerous mediators of cellular and plasma origin with elaborate, interrelated biological effects. The phases of inflammation have been loosely defined as acute, chronic, and resolution. Prostanoids are mediators that have been implicated in all stages of inflammation. Indeed, the inhibition of prostanoid formation by nonsteroid antiinflammatory drugs is the basis for their therapeutic as well as their side effects (1). Prostanoid release is initiated by the liberation of the substrate arachidonic acid, which is metabolized by cyclooxygenase (COX) to prostaglandin H_2 (PGH_2). This is further metabolized by other enzymes to PGs, prostacyclin and thromboxane A_2 (see ref. 2). COX is localized primarily in the endoplasmic reticulum and exists as a dimer of 70-kDa subunits (3). Recently, a second isoform of COX has been described. The constitutive enzyme, COX-1, is present in almost all cell types, whereas COX-2 is induced in a number of cells by proinflammatory stimuli (see ref. 4).

Nitric oxide (NO), formed from L-arginine by NO synthase (NOS; EC 1.14.13.39), is also generated by many cell types (see refs. 5 and 6). Several isoforms of NOS have been isolated, purified, cloned, and expressed (see refs. 5 and 7–10). Endotoxin or cytokines induce macrophages, vascular smooth muscle, and other cells to express an isoform of NOS [inducible NOS (iNOS); calcium-independent] that, through the generation of NO, plays an important role in the cytotoxicity of activated macrophages (see refs. 5 and 11) and contributes to the circulatory failure associated with shock due to sepsis (12–15) or hemorrhage (16).

Here we have investigated the profile of the constitutive and inducible isoforms of COX and NOS in a well-defined air pouch model of granulomatous inflammation (17, 18). COX and NOS activities in normal skin and skin samples from the first 24 h after initiation of the air pouch were measured as an index of activities in the acute stage of inflammation. COX and NOS activities in the granulomatous tissue were measured at 3, 5, 7, 14, and 21 days, covering the chronic and resolving stages of the inflammatory process.

MATERIALS AND METHODS

Initiation of Air Pouch and Preparation of Samples. Female Tuck original mice (30 ± 2 g) were injected with 3 ml of air into the dorsal subcutaneous tissue. Twenty-four hours later (time 0), 0.5 ml of 0.1% (vol/vol) croton oil in Freund's complete adjuvant was injected into the air pouch (18). Over the next 24 h, inflammatory cells migrate into the dermis and accumulate at the edge of the air pouch. At this point a distinct granulomatous tissue has not yet developed. Full-thickness skin biopsies were taken from normal skin, from air pouch skin before injection of the irritant (time 0), and at 6, 12, and 24 h after injection of the irritant. By day 3 the granulomatous tissue is clearly defined, lining dorsal and ventral surfaces of the air pouch. Therefore, granulomatous tissue was dissected from the ventral region of the air pouch at 3, 5, 7, 14, and 21 days as representative samples of chronically inflamed tissue progressing to resolution.

Measurement of COX Activity. Tissues were immediately homogenized at 4°C in Tris buffer (50 mM, pH 7.4) containing phenylmethylsulfonyl fluoride (1 mM), pepstatin A (1.5 mM), and leupeptin (0.2 mM) in a ratio of 5:1 (vol/wt). The protein concentration in the homogenates was measured using the Bradford assay (19) adapted for a 96-well plate reader, using bovine serum albumin as standard. Homogenates were incubated at 37°C for 30 min in the presence of excess arachidonic acid (30 μM). The samples were boiled and centrifuged at 10,000 × *g* for 30 min. The concentration of 6-keto $PGF_{1\alpha}$ or PGE_2 present in the supernatant was measured by radio-

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Abbreviations: COX, cyclooxygenase; NO, nitric oxide; NOS, NO synthase; cNOS, constitutive NOS; iNOS, inducible NOS; TGF-β, transforming growth factor β; PG, prostaglandin; IL, interleukin; PDGF, platelet-derived growth factor; EGF, epidermal growth factor; TNF, tumor necrosis factor.

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immunoassay (radiolabeled prostanoids were obtained from Amersham; antibodies were from Sigma).

Measurement of NOS Activity. NOS activity was measured as the ability of tissue homogenates to convert L-[^{3}H]arginine to L-[^{3}H]citrulline. Samples were prepared as above and incubated at room temperature for 30 min in the presence of NADPH (1 mM), calmodulin (30 nM), tetrahydrobiopterin (5 μM), calcium (2 mM), and a mixture of unlabeled and L-[^{3}H]arginine (10 μM) (20). L-Valine (50 mM), which inhibits the conversion of L-arginine to L-citrulline by arginase and the transcarbamoylase cycle, was included in the reaction mixture to increase the specificity of the assay. In experiments to assess the contribution of the iNOS to the total NOS activity, calcium was replaced with EGTA (1 mM). In addition, L-citrulline formation was determined in the absence of NADPH as a measure of nonspecific conversion of L-arginine. Incubations were terminated by the addition of 1 ml of Hepes (20 mM, pH 5.5) containing 1 mM EGTA and EDTA. The newly formed L-citrulline was separated from L-arginine by passing the reaction mixture over Dowex columns (1 ml), and the eluted labeled material was measured using a Beckman scintillation counter.

Western Blot Analysis. Tissue homogenates were boiled (10 min) with gel loading buffer (50 mM Tris/10% SDS/10% glycerol/10% 2-mercaptoethanol/2 mg of bromophenol blue per ml) in a ratio of 1:1 and centrifuged at 10,000 × *g* for 10 min. Protein concentrations of the supernatants were determined as above, and total protein equivalents for each sample were separated on 10% SDS/polyacrylamide minigels (Hoefer) using the Laemmli buffer system and transferred to poly(vinylidene difluoride) membranes (Millipore). Nonspecific IgGs were blocked with 5% dried milk protein and incubated with specific antibody to COX-2 (Cayman Chemicals, Ann Arbor, MI) used at a dilution of 1:1000. Bands were detected with a horseradish peroxidase-conjugated secondary antibody and developed with diaminobenzidine tetrahydrochloride. Rainbow marker (Amersham) and prestained blue (Sigma) protein markers were used for molecular weight determinations. Statistical analysis was performed using Student's paired *t* test and Mann Whitney U test where appropriate. A P value of <0.05 was taken as significant. All compounds used were obtained from Sigma unless otherwise stated.

RESULTS

COX Activity in Skin Biopsies in the First 24 h after Injection of the Irritant. Full-thickness biopsies from normal skin and air pouch skin taken before injection of the irritant (time 0) formed similar levels of PGE_2 and 6-keto $PGF_{1\alpha}$. Therefore in all experiments the time point 0 includes values for normal skin. Skin biopsies from air pouches at 6, 12, and 24 h after injection of the irritant formed PGE_2 and 6-keto $PGF_{1\alpha}$, peaking at 12 h (Table 1, Fig. 1). Formation of PGE_2 was greater than that of 6-keto $PGF_{1\alpha}$ in all samples.

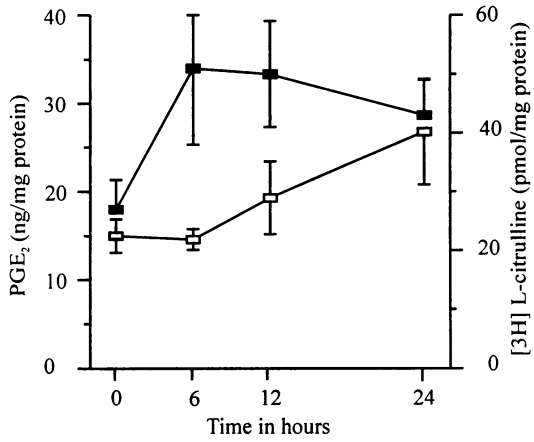

FIG. 1. COX activity, measured by the formation of PGE_2 (□) in normal skin (time 0) and at 6, 12, and 24 h after injection of the irritant. The iNOS activity (■) measured in the absence of calcium in the skin samples is also shown. Data represent the mean ± SEM of 14 determinations from seven separate animals.

COX Activity in Granulomatous Tissue at 3, 5, 7, 14, and 21 Days after Injection of the Irritant. Homogenates of granulomatous tissue contained increasing levels of COX activity, with a significant ($P < 0.05$) peak of activity at 14 days for 6-keto $PGF_{1\alpha}$ and PGE_2. Formation of PGE_2 was greater than that of 6-keto $PGF_{1\alpha}$ in all samples (Table 2, Fig. 2).

Characterization of COX Isoforms Present in the Skin and Granulomatous Tissue. Specific antibodies to COX-2 recognized a band of ≈70 kDa in all samples. In skin samples, COX-2 protein was increased at 6, 12, and 24 h after initiation of the air pouch. Granulomatous tissue at all time points contained more COX-2 protein than skin samples. COX-2 protein levels at 14 and 21 days were higher than at days 3, 5, and 7 (Fig. 3). Barely detectable levels of COX-1 protein were present in samples from all time points after induction of the air pouch (data not shown).

NOS Activity in Skin Biopsies in the First 24 h after Injection of the Irritant. Skin biopsies contained detectable levels of constitutive NOS (cNOS; calcium-dependent) and iNOS activity (Table 1). There was an increase in the amount of iNOS activity at 6, 12, and 24 h after injection of the irritant. The iNOS activity increased to a plateau at 6 h that was sustained at 12 h and 24 h (Table 1, Fig. 1). The cNOS activity accounted for approximately half of the total activity. In the absence of NADPH, the conversion of L-arginine to L-citrulline in skin samples from all points was greatly reduced ($P < 0.05$) (Table 1).

NOS Activity in Granulomatous Tissue at 3, 5, 7, 14, and 21 Days after Injection of the Irritant. NOS activity was present in the granulomatous tissue at considerably higher levels, at

Table 1. COX and NOS activity in skin biopsies in the acute stage of inflammation

Activity	Time, h			
	0	6	12	24
COX (6-keto $PGF_{1\alpha}$)	4.8 ± 0.9	8.5 ± 1.6	11.7 ± 2.0	8.6 ± 0.9
COX (PGE_2)	15 ± 1.9	14.6 ± 1.2	19.3 ± 4.1	26.8 ± 6.0
Total NOS	49 ± 6	64 ± 10	81 ± 15	81 ± 8
iNOS	27 ± 5	51 ± 13	50 ± 9	43 ± 6
NOS − NADPH	10 ± 7	6 ± 3	14 ± 5	5 ± 2

COX and NOS activities in skin were measured before injection of the irritant (time 0) and at 6, 12, and 24 h after injection of the irritant. COX activity was measured by the formation of 6-keto $PGF_{1\alpha}$ and PGE_2 (ng/mg of protein per 30 min). NOS activity (pmol of L-citrulline per mg of protein per 30 min) was measured in the presence (total NOS) or absence (iNOS) of calcium. As a measure of nonspecific conversion of L-arginine, NOS activity was also assessed in the absence of the essential cofactor NADPH. Data represent the mean ± SEM of 14 determinations from seven separate animals.

Table 2. COX and NOS activity in granulomatous tissue in the chronic and resolving stages of inflammation

Activity	Time, days				
	3	5	7	14	21
COX (6-keto $PGF_{1\alpha}$)	12.5 ± 1.9	13.0 ± 1.8	26.1 ± 3	42.1 ± 4.5	21.4 ± 6.2
COX (PGE_2)	15.6 ± 1.5	16.1 ± 2.3	24.4 ± 3	56.3 ± 5.2	38.5 ± 8.3
Total NOS	461 ± 33	311 ± 44	548 ± 38	144 ± 29	338 ± 15
iNOS	346 ± 31	287 ± 35	389 ± 39	82 ± 19	287 ± 11
NOS − NADPH	15 ± 5	8 ± 6	8 ± 6	13 ± 3	0

COX and NOS activities in granulomatous tissue were measured at 3, 5, 7, 14, and 21 days after injection of the irritant. COX activity was measured by the formation of 6-keto $PGF_{1\alpha}$ and PGE_2 (ng/mg of protein per 30 min). NOS activity (pmol of L-citrulline per mg of protein per 30 min) was measured in the presence (total NOS) or absence (iNOS) of calcium. As a measure of nonspecific conversion of L-arginine, NOS activity was also assessed in the absence of the essential cofactor NADPH. Data represent the mean ± SEM of 20 determinations from 10 separate animals.

all time points measured, than in skin samples. The iNOS activity in the granulomatous tissue was >90% of total NOS activity (Table 2, Fig. 2) and conversion of L-arginine to L-citrulline was significantly reduced ($P < 0.05$) in the absence of NADPH (Table 2). The iNOS activity rose to high levels at days 3, 5, and 7. However, activity was reduced substantially at 14 days but returned to a high level at day 21. No significant amounts of NOS activity were present in the spleens or lungs of day 7 animals, a time at which iNOS activity in the granulomatous tissue was maximal.

DISCUSSION

The histology of the granulomatous tissue in the croton oil-induced chronic model of inflammation in the mouse has been extensively described (17, 18). In the acute stage of inflammation, during the first 24 h, the air pouch skin contains large numbers of polymorphonuclear neutrophils. As the granulomatous tissue develops over the next 2 or 3 days, the cellular population changes to be dominated by macrophages. Fibroblasts and endothelial cells subsequently migrate into the newly forming tissue, resulting in a fibrotic and highly vascularized granuloma, which ultimately resolves over several months.

Here we have shown that inducible isoforms of COX and NOS are elevated at all time points measured in this model. In the acute stage, NOS activity in the skin was raised at 6 h and sustained up to 24 h. In contrast, the increase in COX activity was slower in onset and was maximal at 24 h. Over this time course there was an approximate 2-fold increase above basal levels in COX and NOS activity. These findings support the well-established role for PGs as mediators of the acute inflammatory response (see ref. 21) and allow a similar interpretation for the role of NO.

However, the role of NO in inflammation is not clear, as both pro- and antiinflammatory actions have been reported. NO is a potent vasodilator and contributes to edema formation in acute inflammation (22). The edema induced by substance P is mediated through the release of NO (23). Furthermore, NO has been implicated in the increased vascular permeability associated with acute immune complex inflammation (24). A proinflammatory role for NO in chronic inflammation has been inferred, based on the observation of elevated levels of nitrite in rheumatoid synovial fluid (25). In addition, adjuvant-induced arthritis in rats was exacerbated by L-arginine and suppressed by an NOS inhibitor, interpreted as a modulation by NO of the activity of T lymphocytes and/or macrophages (26). Antiinflammatory actions of NO are demonstrated by its ability, when released from endothelial cells, to inhibit platelet and leukocyte adhesion (27–29) and platelet aggregation (ref. 30; for review of NO in inflammation, see ref. 31).

In the granulomatous tissue, taken as an indication of chronic inflammation, maximal iNOS activity was between 3 and 7 days, whereas COX activity continued to rise until day 14. This pattern of COX and NOS induction between 3 and 21 days suggests that there is differential regulation of the enzymes. This may be attributed to the changing profile of cytokines as the inflammatory process progresses. COX-2 is induced in a number of cell types by epidermal growth factor (EGF; ref. 32), basic fibroblast growth factor (33), platelet-derived growth factor (PDGF; ref. 34), interleukin 1 (IL-1; refs. 35 and 36), and transforming growth factor β (TGF-β; ref. 37). Tumor necrosis factor α (TNF-α) also increases COX mRNA in Swiss 3T3 fibroblasts (38) and synergizes with IL-1

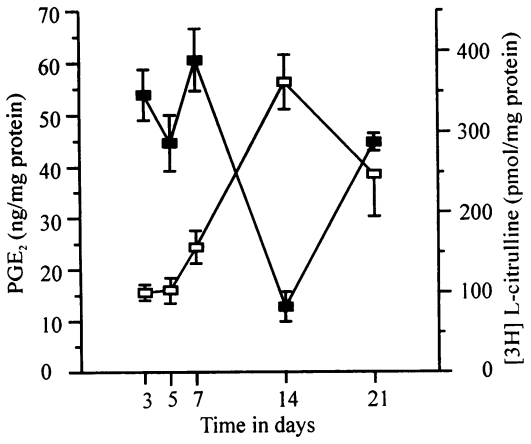

FIG. 2. COX and NOS activities in granulomatous tissue at different times after injection of the irritant. COX activity was measured by the formation of PGE_2 (□) at 3, 5, 7, 14, and 21 days. The iNOS activity (■) measured in the absence of calcium in the granulomatous tissue is also shown. Data represent the mean ± SEM of 20 determinations from 10 separate animals.

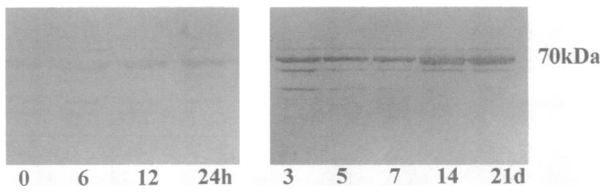

FIG. 3. Levels of COX-2 protein in extracts from normal skin (time 0) and air pouch skin biopsies at 6, 12, and 24 h and in granulomatous tissue at 3, 5, 7, 14, and 21 days after injection of the irritant. The specific antibody to COX-2 recognized a protein that migrated at ≈70 kDa when compared to protein standards. Similar results were seen using tissue extracts from two other animals for each time point. Western blots primed with a selective antibody to COX-1 showed low levels of protein (70 kDa) that remained constant throughout the depicted time course (data not shown).

to stimulate PG production (39). Similarly, NOS activity is induced by cytokines such as IL-1 (40) and TNF-α (41) but inhibited by TGF-β (42). Appleton *et al.* (18) showed that the levels of IL-1α and IL-1β, EGF, and TNF-α are greatest in the early stages of the formation of the murine air pouch. All of these cytokines, either alone or in combination, are, therefore, possible candidates for the early induction of COX and NOS in the acute phase of inflammation. TGF-β is known to induce COX but inhibit NOS (37, 42). Furthermore, TGF-β immunoreactivity is greatest at 14 days in this model (18). Thus, the rise in COX activity and fall in NOS activity at 7–14 days may be due to the presence of TGF-β (see Fig. 4).

The release of large amounts of NO by iNOS can inhibit the induction of COX-2 (43) and suppress the formation of COX metabolites (44, 45). In contrast, low levels of NO may activate COX (44, 46, 47). Here we have shown that between days 3 and 7 elevated NOS activity is associated with a fall in COX activity. When COX levels were maximal at day 14, NOS activity was depressed (see Fig. 2). Thus, the modulatory actions of NO on COX activity may also contribute to the dissociation of activities seen in the chronic phase of inflammation. Interestingly, PGE_2 inhibits endotoxin-stimulated release of NO in J774 macrophages (48) so that PGs may also modulate NOS activity.

The cardinal signs of acute inflammation as described by Celsus (30 B.C. to A.D. 38) were heat, redness, swelling, and pain. It is well accepted that PGE_2 formed by COX contributes to these signs of acute inflammation by causing vasodilatation, increased vascular permeability, edema, and pain. The relative contributions of COX-1 and COX-2 to this PG release are not yet determined. Interestingly, indomethacin inhibits carrageenin edema in the rat paw when given before the carrageenin but not when given 2 hr afterward (49).

In chronic inflammation, where the dominant inflammatory cell is the macrophage, which contains COX-2, PGE_2 may additionally exert a proinflammatory effect by contributing to angiogenesis, a process vital to the maintenance of newly formed granulomatous tissue (see ref. 50). However,

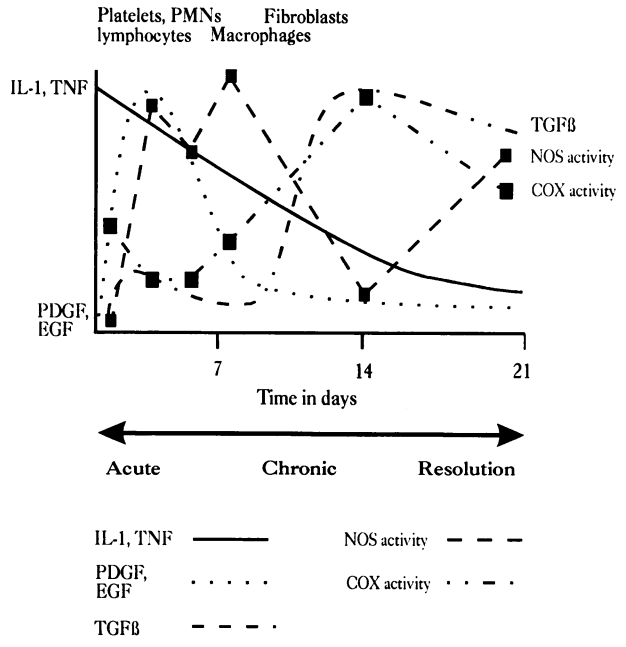

FIG. 4. Relative levels of cytokine immunolabeling, iNOS and COX activity (PGE_2 formation), and inflammatory cell influx during the development of granulomatous tissue in the murine air pouch [modified and reprinted with permission from ref. 18 (copyright The United States–Canadian Academy of Pathology)]. PMNs, polymorphonuclear neutrophils.

an antiinflammatory role for "PGs," acting through their cell surface receptors to raise cAMP, has been championed by Weissmann (51). Certainly, *in vitro*, PGE_2 inhibits the production of cytokines (52–54) and cytokine-induced proliferation in a number of cell types (55–57). *In vivo*, however, the actions of PGs are more complex, in that COX inhibitors exacerbate cartilage erosion but reduce bone loss (see ref. 58). Hopefully, the controversial aspects of the contributions of PGE_2 and other PGs to the chronic inflammatory response and the resolution of inflammation will be clarified by the use of selective inhibitors of COX-1 and COX-2. In addition, selective inhibitors of cNOS and iNOS will also allow a better understanding of the roles of NO in inflammation.

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