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SHORT COMMUNICATION

Relationship Between Nitric Oxide and Prostaglandins in Carrageenin Pleurisy

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ABSTRACT. The correlation between endogenous nitric oxide (NO) generation and prostaglandin biosynthesis was studied in rat carrageenin pleurisy induced by the injection of 0.2 mL of 1% λ-carrageenin into the pleural cavity. The pleural exudate was collected at 4 hr and the amounts of NO_2^- + NO_3^- (NOx) and prostaglandin E_2 (PGE_2) measured. The NOx present in the inflammatory exudate was determined by measuring the NO_2^- with the Griess reaction, after the reduction of NO_3^- to NO_2^- using acid-washed cadmium powder. PGE_2 was measured by radioimmunoassay. The NO synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME; 1-3-10 mg/kg subcutaneously) reduced NOx by 20 ± 7%, 41 ± 6% and 55 ± 9% ($P < 0.01$) and PGE_2 by 9 ± 6%, 41 ± 11% and 74 ± 9% ($P < 0.001$). Conversely, L-arginine (300 mg/kg SC) increased NOx by 39 ± 7% ($P < 0.01$) and PGE_2 by 78 ± 6% ($P < 0.001$). The NO scavenger haemoglobin (Hb), coinjected into the pleural cavity (3 mg/site) with carrageenin, produced a parallel inhibition of NOx (65 ± 16%, $P < 0.001$) and PGE_2 (71 ± 18%, $P < 0.001$). The soluble guanylate cyclase inhibitor methylene blue (Mb; 2 mg/site) had no effect. Moreover haemoglobin, but not methylene blue, was able to significantly suppress the L-arginine-induced increase of both NOx and PGE_2. In each pleural exudate, independently from the animal treatment, the amount of NOx was highly correlated to the amount of PGE_2 ($r = 0.93$, $P < 0.001$). These results suggest that in rat carrageenin pleurisy the modulation of the L-arginine:NO pathway results in a parallel modulation of prostaglandin biosynthesis. The interaction between cyclooxygenase and the NO pathway may represent an important mechanism for the modulation of the inflammatory response. BIOCHEM PHARMACOL **55**;7:1113–1117, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. carrageenin pleurisy; inflammation; nitric oxide; prostaglandins

The biosynthesis of nitric oxide (NO) from L-arginine is a pathway for the regulation of cell function and communication [1]. The synthesis of NO from L-arginine in mammalian cells is catalyzed by several isoforms of the enzyme NO synthase. The isoforms of NO synthase are currently classified as either constitutive or inducible. The constitutive isoforms, found in endothelial cells and some neurons, are Ca^{2+}/calmodulin-dependent, whereas the inducible NO synthase found in macrophages and other cell types is Ca^{2+}/calmodulin-independent. The NO generated by the constitutive NO synthase acts as a transduction mechanism for the regulation of several physiological responses (e.g. vasodilation and neurotransmission) through the activation of the soluble guanylate cyclase. The NO synthetized by the inducible NO synthase plays a key role in host defence mechanisms, as a cytotoxic molecule for invading microorganisms and tumor cells, and is involved in pathological vasodilation and tissue damage [1].

We have shown that NO is involved in acute inflammation since rat paw oedema induced by carrageenin, a type of acute inflammation mainly sustained by prostaglandin release [2], is increased by L-arginine and decreased by NO synthase inhibitors [3]. NO appears to modulate oedema formation not only by increasing local blood flow but also by stimulating prostaglandin formation at the inflammation site. We have demonstrated that the amounts of vasodilator prostaglandins formed in the rat paw injected with either arachidonic acid or carrageenin are reduced by NO synthase inhibitors and increased by both endogenous or exogenous NO [4, 5]. Furthermore, we have previously shown that prostacyclin biosynthesis in the lung of endotoxin treated-rats is increased by L-arginine and decreased by NO synthase inhibitors [6]. Thus, endogenous NO seems to play a relevant role in the modulation of prostaglandin generation at the inflammation site, probably by activating cyclooxygenase through a cGMP-independent mechanism [4, 5, 7]. Since in rat carrageenin pleurisy the inducible isoforms of both NO synthase and cyclooxygenase pathways are expressed [8, 9], we have investigated in this model of inflammation the correlation occurring *in vivo* between endogenous NO generation and prostaglandin E_2 biosynthesis.

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TABLE 1. Modulation of L-arginine:NO pathway in rat carrageenin pleurisy

Group	Treatment	Exudate (mL)	Cells ($\times 10^6$)	NOx (nmol)	PGE2 (pg)	*n*
1	Control	1.05 ± 0.03	97 ± 4	65.6 ± 3.7	321 ± 25	12
2	L-NAME 1 mg/kg	0.82 ± 0.05	94 ± 4	52.7 ± 3.6	292 ± 18	4
3	L-NAME 3 mg/kg	0.67 ± 0.04	72 ± 3	39.8 ± 2.4	190 ± 22	4
4	L-NAME 10 mg/kg	0.40 ± 0.09‡	56 ± 6‡	29.6 ± 2.9†	83 ± 11‡	9
5	Hb 3 mg/site	0.29 ± 0.11‡	70 ± 4†	23.1 ± 3.9‡	92 ± 17‡	6
6	Mb 2 mg/site	1.02 ± 0.01	101 ± 7	61.3 ± 9.4	390 ± 15	6
7	L-arginine 300 mg/kg	1.37 ± 0.09*	118 ± 4*	91.3 ± 6.2†	570 ± 35‡	12
8	L-arginine 300 + Hb 3	0.80 ± 0.05§	69 ± 10§	49.4 ± 2.7§	250 ± 42§	6
9	L-arginine 300 + Mb 2	1.34 ± 0.17	115 ± 7	85.8 ± 12.9	580 ± 58	6

N^G-nitro-L-arginine methyl ester (L-NAME) and L-arginine were injected SC 1 hr before carrageenin. Haemoglobin (Hb) and methylene blue (Mb) were injected into the pleural cavity concomitantly with carrageenin. Results are expressed per rat and represent the mean ± SEM of *N* animals.

*$P < 0.05$.

†$P < 0.01$.

‡$P < 0.001$ vs control group.

§$P < 0.001$ vs L-arginine group.

MATERIALS AND METHODS

Carrageenin-Induced Pleurisy

Male Wistar rats (180–200 g) were slightly anesthetized with ether, and 0.2 mL of 1% λ-carrageenin, suspended in sterile saline solution, were injected into the right pleural cavity (control group). Groups of animals were treated subcutaneously (SC) 1 hr prior to carrageenin injection with the NO synthase inhibitor L-NAME† (1-3-10 mg/kg) or L-arginine (300 mg/kg). The pleural cavities of other groups were injected, concomitantly with carrageenin, with 3 mg of the NO scavenger Hb or 2 mg of the soluble guanylate cyclase inhibitor Mb. In some experiments, the animals treated with L-arginine were also injected with Hb or Mb into the right pleural cavity as reported above. Animals were sacrificed 4 hr after the induction of pleurisy in an atmosphere of CO_2. Pleural exudate from each animal was harvested by washing the pleural cavity with 2 mL of sterile saline solution containing 5 U/mL of heparine and 10 μg/mL of indomethacin. The exudate was centrifuged at 800 *g* for 10 min and cell pellet resuspended in saline. Total cell count was estimated after Trypan blue staining, using the Burker counting chamber. All products were from Sigma.

NOx Assay

The amount of NOx present in the inflammatory exudate was determined according to Thomsen *et al.* [10]. After reducing NO_3^- to NO_2^- using acid-washed cadmium powder (Aldrich), NO_2^- was measured using a microplate assay method based on the Griess reaction.

Radioimmunoassay of PGE_2

PGE_2 in the supernatant of centrifuged exudate was assayed by radioimmunoassay as previously described [5]. The antibody anti-PGE_2 was kindly given by Dr. G. Ciabattoni (Catholic University, Rome, Italy).

Statistics

Data are expressed as mean ± SEM. Comparisons were made using the Bonferroni multiple comparison test. The level of statistically significant difference was defined as $P < 0.05$.

RESULTS

Exudate Volume and Leucocyte Migration

Injection of 0.2 mL of 1% λ-carrageenin into the pleural cavity of rats caused an accumulation of the inflammatory exudate. As shown in Table 1, the average volume of exudate in control animals (carrageenin only) was 1.05 ± 0.03 mL per rat ($N = 12$) and the total leucocyte number migrated into the pleural cavity was 97 ± 4 × 10^6 per rat ($N = 12$). Pretreatment of rats with L-NAME (1-3-10 mg/kg, SC) 1 hr prior to carrageenin injection caused a dose-dependent inhibition of both exudate volume and total leucocyte number compared to the control group (Table 1). At the dose of 10 mg/kg, the volume of the exudate was significantly reduced by 62% ($P < 0.001$; $N = 9$), while the number of cells migrated into the pleural cavity decreased by 42% ($P < 0.001$; $N = 9$). Conversely, L-arginine (300 mg/kg, SC) 1 hr prior to carrageenin injection significantly increased both exudate volume (+30%, $P < 0.05$; $N = 12$) and leucocyte number (+22%, $P < 0.05$; $N = 12$). The injection into the pleural cavity Hb (3 mg) significantly reduced either the exudate volume (−72%, $P < 0.001$; $N = 6$) or the cell number (−28%, $P < 0.01$; $N = 6$). Treatment with Mb (2 mg/site; $N = 6$) was without any effect on either exudate volume or the number of migrated cells. Moreover, the increases in exudate volume and leucocyte number induced by L-arginine were both suppressed by 42% ($P < 0.001$; $N = 6$) by Hb, whereas Mb ($N = 6$) had no effect.

† *Abbreviations:* Hb, haemoglobin; L-NAME, N^G-nitro-L-arginine methyl ester; Mb, methylene blue; NO, nitric oxide; NOx, NO_2^- + NO_3^-; PGE_2, prostaglandin E_2.

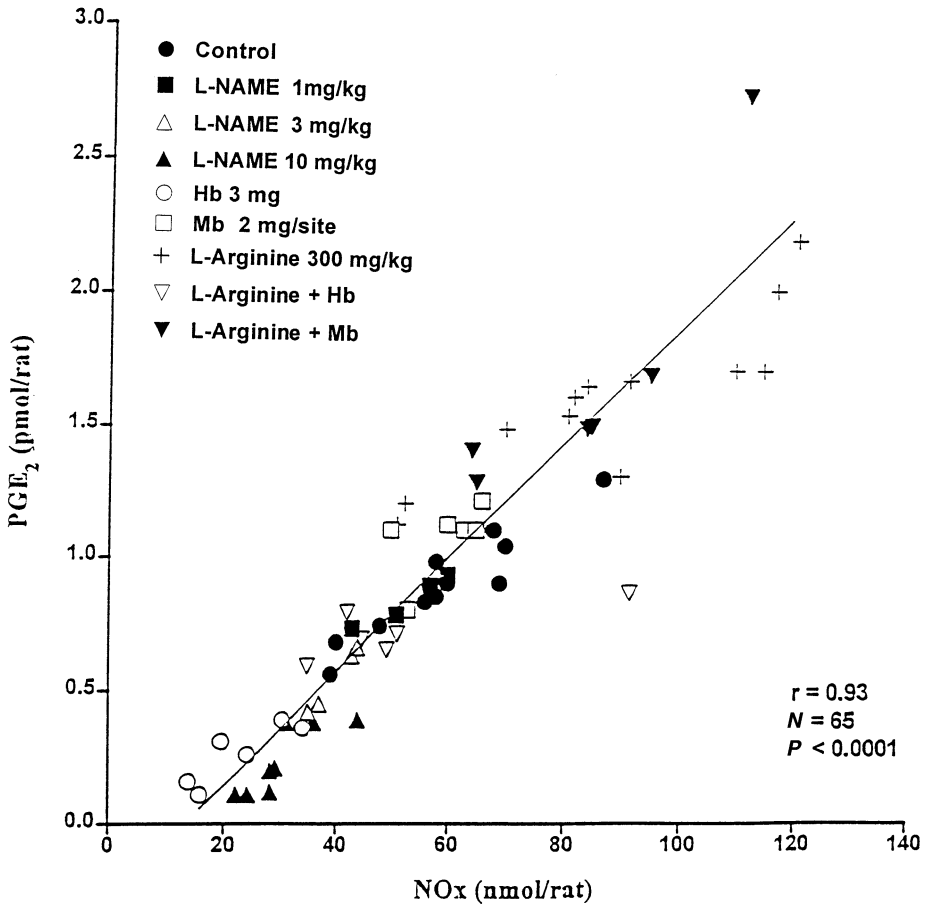

FIG. 1. Correlation between the amounts of NO_2^- + NO_3^- (NOx) and prostaglandin E_2 (PGE_2) in carrageenin-induced pleural exudate of each individual rat treated as shown in Table 1.

NOx and PGE_2 in Pleural Exudate

The pleural exudate of carrageenin-injected rats contained a detectable amount of either PGE_2 (321 ± 25 pg/rat, N = 12) or NOx (65.6 ± 3.7 nmol/rat, N = 12). As shown in Table 1, the amount of both NOx and PGE_2 measured in the pleural exudate was correlated with the severity of the inflammatory process. L-NAME at the doses of 1-3-10 mg/kg/SC caused a dose-dependent inhibition of both NOx and PGE_2. At the dose of 10 mg/kg, the amount of NOx was significantly reduced by *ca.* 55% ($P < 0.01$; $N = 9$), while PGE_2 decreased by *ca.* 74% ($P < 0.001$; $N = 9$). L-arginine (300 mg/kg/SC) significantly increased NOx by 39% ($P < 0.01$; $N = 12$) and PGE_2 by 78% ($P < 0.001$; $N = 12$). When Hb (3 mg/site) was injected concomitantly with carrageenin, a significant inhibition of NOx (−65%, $P < 0.001$; $N = 6$) and PGE_2 (−71%, $P < 0.001$; $N = 6$) was observed. Conversely, Mb (2 mg/site; $N = 6$) did not produce any effect. Hb, but not Mb, was also able to significantly suppress the L-arginine-induced increase in the amount of both NOx (−46%, $P < 0.001$; $N = 6$) and PGE_2 (−56%, $P < 0.001$; $N = 6$) in the pleural exudate. Thus, any increase or decrease in NOx levels corresponded to a parallel increase or decrease in the amount of PGE_2, indicating a strict correlation between the two parameters. The analysis of this correlation is shown in Fig. 1, where the amount of NOx found in the exudate of each individual animal and the corresponding amount of PGE_2 have been plotted. These data indicate that NOx and PGE_2 present in the pleural exudate are highly correlated ($r = 0.93$; $P < 0.001$; $N = 65$), independently from the animal treatment.

DISCUSSION

In this study, we have shown that the modulation of the L-arginine:NO pathway in rat carrageenin-induced pleurisy results in a parallel modulation of prostaglandin biosynthesis at the inflammation site. We found that any increase or decrease in the amounts of NOx in the pleural exudate was highly correlated to a concomitant increase or decrease in PGE_2. However, such a correlation appears less evident when the concentrations of NOx and PGE_2 in the pleural exudate, rather than the amounts, are considered (data not shown). A lack of correlation is in fact observed with those treatments (e.g. Hb 3 mg/site, L-NAME 10 mg/kg) which produce a dramatic reduction in exudate volume not associated with a comparable inhibition of NOx and PGE_2 production. In any case, it should be mentioned that the

amounts of the metabolite in inflammatory fluids are currently referred to the volume of exudate recovered and not calculated as concentration [11–13]. The amount of the prostanoid in the pleural exudate was decreased by the NO synthase inhibitor L-NAME as well as by the NO scavenger. These results are in agreement with previous data showing that NO synthase inhibitors decrease prostaglandin biosynthesis in other models of acute inflammation [4, 5, 14]. Moreover, the NO synthase inhibitor reduced both the volume of pleural exudate and the number of leucocytes migrated into the pleural cavity, suggesting a modulatory role for NO in the inflammatory process as shown in other models of inflammation [3, 15–17]. The ability of NO inhibitors to modulate leucocyte migration is controversial, since these compounds have been shown to increase [18, 19], decrease [20] or not affect [21] cell accumulation at the inflammation site. However, the contribution of emigrated leucocytes to the production of prostaglandins at the inflammation site does not seem relevant, since in the rat carrageenin air pouch a 98% reduction in cell infiltration, produced by treating animals with colchicine, did not affect the amount of PGE_2 in the fluid exudate [20]. Moreover, although our results point toward a stimulatory effect of NO on prostaglandin production, it is important to recognize that the NO synthase inhibitor L-NAME may elevate blood pressure and reduce blood flow in different organs [22].

L-arginine, the substrate for NO generation, greatly enhanced the inflammatory reaction (exudate volume and cell migration) as well as the amounts of PGE_2 released into the pleural cavity. Enhanced PGE_2 production seems to be dependent on increased formation of NO, as suggested by the concomitant increase in the levels of NOx found in the pleural exudate. NO is strongly generated by the inducible NO synthase, which is the predominant isoform in carrageenin-induced pleurisy [8] and whose activity is stimulated by an excess of substrate. However, it is important to recognize that L-arginine may increase vasodilation and vascular permeability at the inflammatory site, as we have observed in another model of inflammation [3].

Our results suggest that the action of NO on prostaglandin biosynthesis seems to be mediated by a cGMP-independent mechanism, since the soluble guanylate cyclase inhibitor Mb did not reduce the stimulatory effect of NO on the release of PGE_2. The inefficacy of Mb was not due to the dose or the way of administration used, because the same dose of this agent, administered by local injection, was highly effective in reducing the SIN-1-induced rat paw oedema, which depends on the release of exogenous NO acting through a cGMP-dependent mechanism [4]. However, the actual role of cGMP in the NO-dependent stimulation of prostaglandin biosynthesis should be further elucidated by using more permeable or selective inhibitors of soluble guanylate cyclase such as 6-anilo-5,8-quinolinequinone [23] or 1H-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one [24]. Substantial evidence reported by us and others, both *in vitro* [6, 7, 25, 26, 27] and *in vivo* [4, 5, 20, 28, 29], has demonstrated that NO stimulates prostaglandin biosynthesis. It has been suggested that NO stimulates cyclooxygenase by interacting directly with its iron-haeme centre [7]. However, as recently reviewed, the actual mechanism by which NO apparently increases cyclooxygenase activity may depend on other effects of NO on the enzyme, e.g. allosteric regulation, prevention of self-deactivation [30] or, as recently suggested by Hajar *et al.* [31], the possibility that NO might increase prostaglandin biosynthesis by S-nitrosation of the cysteine residues in the catalytic domain of the cyclooxygenase enzyme. The "cross talk" occurring between the L-arginine:NO and cyclooxygenase pathways, particularly at the level of the inflammatory response, appears to be bidirectional, although the effect of prostaglandins on NO production, possibly related to an elevation in intracellular cAMP, is controversial (for review see [30]).

In conclusion, although the influence of NO on prostanoid biosynthesis requires further elucidation, the interaction between cyclooxygenase and the NO pathway may represent an important mechanism for the modulation of the inflammatory response.

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