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Endogenous nitric oxide inhibits leukotriene B_4 release from rat alveolar macrophages

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Abstract

Effects of endogenous nitric oxide (NO) on the release of mediators of the lipoxygenase and cyclo-oxygenase pathway from rat alveolar macrophages were studied. Alveolar macrophages, freshly isolated or after 18-h culture, were incubated in (amino acid-free) Krebs medium and labelled with [^{3}H]arachidonic acid. The release of [^{3}H]leukotriene B_4 and [^{3}H]prostanoids (separated by high performance liquid chromatography) was determined. A 23187 was used as stimulus, as rising intracellular Ca^{2+} activates directly the phospholipase A_2 and lipoxygenase pathway. A 23187 (10 μM) enhanced [^{3}H]leukotriene B_4 release from freshly prepared alveolar macrophages about 65-fold, but only 5- to 6-fold from cultured alveolar macrophages. Evoked [^{3}H]leukotriene B_4 release and spontaneous [^{3}H]prostanoid release were inhibited when L-arginine (300 μM) was added to the Krebs incubation medium of alveolar macrophages, in which marked NO synthase had been induced by culture with lipopolysaccharides (10 μg/ml). Inhibitory effects of L-arginine were prevented by N^G-monomethyl-L-arginine (L-NMMA, 100 μM). Inhibition of NO synthase during the culture period by L-NMMA (culture medium, in contrast to Krebs medium, already contains the substrate ofNO synthase, L-arginine), resulted in attenuation of the 'culture-dependent' decline of the evoked release of [^{3}H]leukotriene B_4 and allowed lipopolysaccharides to cause an increase in spontaneous [^{3}H]prostanoid release (i.e., to induce cyclo-oxygenase activity). In conclusion, in rat alveolar macrophages, endogenous NO appears to inhibit the release of mediators of the cyclo-oxygenase and lipoxygenase pathway through multiple sites of action.

Keywords: Alveolar macrophage; Nitric oxide (NO); Lipoxygenase; Cyclooxygenase; Leukotriene B_4

1. Introduction

Alveolar macrophages are part of the non-specific defense mechanisms of the respiratory tract. In addition they also play an important role in the local control of inflammatory reactions and specific immune responses (see Thepen and Havenith, 1994). Alveolar macrophages can appear in different functional states and accordingly release various mediators, such as different cytokines (e.g., tumor necrosis factor-α, interleukin-1) and several lipid mediators (platelet-activating factor, different prostanoids and leukotriene B_4). In addition, an inducible form of nitric oxide (NO) synthase can be induced by various stimuli, including lipopolysaccharides and several pro-inflammatory cytokines (interferon-γ, tumor necrosis factor-α and interleukin-1), resulting in the release of large amounts of NO (Jorens et al., 1991; Nozaki et al., 1993; Hey et al., 1995; Wang et al., 1995).

NO, generated in alveolar macrophages, may exert several functions (see Moncada et al., 1991; Barnes, 1993; Gaston et al., 1994; Xie and Nathan, 1994). There is good evidence that it is an essential effector molecule in non-specific defense mechanisms, but it may also play a role in the signalling between alveolar macrophages and other cells within the airway wall, for example suppressing the TH1 cells (see Barnes and Liew, 1995). Finally, NO could also act as an intracellular messenger within the alveolar

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macrophages themselves. Thus, in murine alveolar macrophage cell lines, it was observed that low levels of NO facilitated prostaglandin release by direct stimulation of cyclo-oxygenase activity (Salvemini et al., 1993; Swierkosz et al., 1995). On the other hand, high levels of NO can suppress prostaglandin release, by inhibiting both the activity and the expression of cyclo-oxygenase-2 (Swierkosz et al., 1995).

It has been reported that patients with bronchial asthma exhale much more NO than do normal human subjects (e.g., Kharitonov et al., 1994; Persson et al., 1994; Massaro et al., 1995) and that this enhanced NO exhalation is reduced after treatment with glucocorticoids, suggesting the involvement of an inducible form of NO synthase. Alveolar macrophages and the bronchial epithelium are considered as cellular compartments in which the expression of this inducible NO synthase may occur (see Barnes and Liew, 1995). In the airways, as in other tissues, NO appears to have multiple effects (see Jorens et al., 1991; Barnes, 1993; Gaston et al., 1994) and in asthmatic patients, some might be beneficial and others, deleterious. It has been suggested that the relatively large amounts of NO generated by inducible NO synthase may promote the inflammatory reactions in the airways of asthmatic patients and therefore worsen the disease, whereas NO generated by the constitutively expressed endothelial and neuronal NO synthase may exert beneficial actions. Therefore, it has been proposed that selective inhibitors of inducible NO synthase could be a new class of antiasthmatic drugs (Barnes and Liew, 1995).

On the other hand, it has been demonstrated that NO can inhibit the activity of soybean lipoxygenase (Kanner et al., 1992). Since leukotriene B_4 is a potent pro-inflammatory mediator of alveolar macrophages, the question arose whether endogenous NO generated in alveolar macrophages might also inhibit the lipoxygenase pathway (i.e., leukotriene B_4 synthesis) in these cells. Such an effect of endogenous NO would indicate that inducible NO synthase could also possess anti-inflammatory potential. Therefore, in the present experiments it was tested whether induction and/or inhibition of inducible NO synthase in isolated rat alveolar macrophages would affect the release of leukotriene B_4. As the 5-lipoxygenase pathway in alveolar macrophages can be activated by a rise in intracellular Ca^{2+}, the Ca^{2+} ionophore, A 23187, was used to stimulate directly leukotriene B_4 release. A radiotracer technique was used, i.e., the release of [^{3}H]leukotriene B_4 was studied after the cells had been labelled with [^{3}H]arachidonic acid. An advantage of this technique is that the release of other ^{3}H-labelled arachidonic acid metabolites (such as prostanoids) can be determined simultaneously. This was of interest, as NO had already been shown to modulate the activity of cyclo-oxygenase in different macrophages cell lines (see above).

Preliminary reports of the present experiments have been given (Brunn et al., 1995a; Racké et al., 1995).

2. Materials and methods

2.1. Materials

A 23187 (Calbiochem, Bad Soden, Germany); amphotericin B (Sigma, Munich, Germany); acetylsalicylic acid (Sigma); [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid (specific activity 7.59 TBq/mmol, Amersham Buchler, Braunschweig, Germany); D-arginine HCl (Sigma); L-arginine HCl (Sigma); Dulbecco's phosphate-buffered saline without Ca^{2+} and Mg^{2+} (D-PBS, Sigma); Dulbecco's modified Eagle's medium/Ham's F-12 medium (DMEM/F12 medium, ICN Biomedicals, Meckenheim, Germany); fetal calf serum (Vitromex, Vilshofen, Germany); ICI 207968 (2-(3-pyridylmethyl)-indazolinone, ICI Pharmaceutical, Mereside, UK); lipopolysaccharides from *Escherichia coli* 0127:B8 (Sigma); L-glutamine (Sigma); May-Grünwald stain (Sigma); N^G-monomethyl-L-arginine (L-NMMA, Sigma); penicillin-streptomycin solution (Sigma).

2.2. Preparation and incubation of alveolar macrophages

Female Sprague-Dawley rats weighing 200–250 g (own breeding) were kept at constant temperature (21°C) and with a regular light (06.30–19.30 h)–dark (19.30–06.30 h) cycle with food and water ad libitum. They were killed by stunning followed by exsanguination. Lung and trachea were excised en bloc, washed with D-PBS and lavaged twice by instilling 15 ml of cold (4°C) D-PBS (Hey et al., 1995). The lavage fluids from 6–8 lungs were pooled and centrifuged at $400 \times g$ for 5 min. The cells were washed three times with D-PBS and thereafter resuspended in DMEM/F12 medium supplemented with 5% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 5 μg/ml amphotericin B and plated (3×10^6 cells/well) in sterile 6-well culture dishes (Nunc, Wiesbaden, Germany). The cells were allowed to adhere for 2 h (37°C, 5% CO_2) before the removal of non-adherent cells by washing with prewarmed medium (37°C) three times at 1-h intervals. Thus, the total adherence protocol lasted for 4 h. The adherent cells consisted of more than 95% macrophages according to morphological criteria (May Grünwald-Giemsa staining).

After this adherence protocol, in most experiments, the cells were cultured for 18 h in the absence or presence of lipopolysaccharides (10 μg/ml) and different further test substances as indicated. Thereafter, the culture medium was removed and the cells were incubated for 2 h in 1.5 ml Krebs-HEPES solution of the following composition (mM): NaCl 118.5, KCl 5.7, $CaCl_2$ 1.25, $MgCl_2$ 1.2, Na_2EDTA 0.03, (+)-ascorbic acid 0.06, HEPES 20.0 and phosphate buffer 1.0 (both adjusted to pH 7.4) and D-glucose 11.1. The medium contained in addition 370 kBq (approx. 30 nM) [^{3}H]arachidonic acid. After this labelling procedure the cells were washed five times within 25 min. Then the cells were incubated in 1.5 ml Krebs-HEPES solution for

two subsequent 50-min periods. During the second incubation period the Ca^{2+} ionophore, A 23187 (10 μM), was added. The incubation media were collected and stored at −80°C until analysed by high performance liquid chromatography (HPLC).

In one series of experiments, the 18-h culture period was omitted. Directly after the adherence protocol, the cells were labelled with [^{3}H]arachidonic acid followed by the 'release' protocol (as described above).

Cell viability was assessed regularly by trypan blue exclusion, both after the adherence protocol as well as after the culture period, and was always greater than 95%.

In some experiments, at the end of the incubation the alveolar macrophages were extracted in 1.5 ml methanol for 12 h at 4°C and the radioactivity in the extraction medium was determined.

2.3. HPLC analysis and flow scintillation spectrometry

[^{3}H]Arachidonic acid metabolites were separated by gradient reverse-phase HPLC. The HPLC system consisted of a gradient pump (Waters 600 E Powerline), equipped with a Rheodyne injection valve (1000 μl loop) and a reverse-phase column (length 250 mm, inner diameter 46 mm, prepacked with Shadon ODS-Hypersil, 5 μm). The mobile phase was continuously pumped through the column at a rate of 1 ml/min. The effluent was directly diverted to a flow scintillation spectrometry system (see below). The solvent system used (a methanol/glacial acid gradient) had previously been shown to allow the separation of all classes of eicosanoids (Henke et al., 1984; Van Scott et al., 1990, Brunn et al., 1995b). Two solvents, A (100% methanol) and B (10% methanol buffered to pH 5.05 with glacial acid and ammonium hydroxide) were mixed to yield four gradients over 108 min (52, 62, 71 and 100% solvent A, changing at 27, 52, 77 min). ^{14}C- or ^{3}H-labelled standards were used to identify the retention time of the various arachidonic acid metabolites. Examples of radiochromatograms of samples collected during rest and stimulation, respectively, are given in Fig. 1. Arachidonic acid eluted after about 88 min and leukotriene B_4 after about 47 min. Various prostanoids (prostaglandin E_2, prostaglandin D_2 and thromboxane A_2) eluted, poorly separated, between about 15 and 20 min. In the present study, this 'prostanoid fraction' was pooled and taken as a measure of cyclo-oxygenase activity. None of the drugs tested in the present study interfered with the HPLC when added to solutions of labelled standards of the prostanoids, leukotriene B_4 or arachidonic acid.

Radioactivity in the HPLC eluent was determined with a flow scintillation spectrometry system (Radiomatic FLO-ONE\Beta A 500 detector, Canberra Packard). The HPLC effluent was automatically mixed with a flow scintillation cocktail (AP-FLOW, Canberra Packard) and the radioactivity was continuously monitored. External standardization was used to determine the counting efficiency for the different gradient conditions (28–32%) and the respective values were used to correct the radioactivity measurement automatically. Radioactivity was finally expressed as disintegrations per minute (dpm).

2.4. Calculations and statistical analysis

The amounts of [^{3}H]prostanoids (see above), [^{3}H]leukotriene B_4 and [^{3}H]arachidonic acid released into the incubation media were expressed as dpm per 3×10^6 cells. The mean values of n observations are given ± S.E.M. The statistical significance of differences was evaluated by paired t-test or Mann-Whitney U-test, as appropriate, using the computer program GraphPad InStat. $P < 0.05$ was accepted as being significant.

3. Results

3.1. Release of [^{3}H]arachidonic acid metabolites from rat alveolar macrophages and effects of culture

Radiochromatograms of incubation media of freshly prepared alveolar macrophages labelled with [^{3}H]arachidonic acid showed several fractions of [^{3}H]arachidonic acid metabolites (Fig. 1). [^{3}H]Leukotriene B_4, [^{3}H]prostanoids and [^{3}H]arachidonic acid were major peaks. The spontaneous rate of release was relatively low and amounted to 203 ± 79, 405 ± 129 and 1323 ± 777 dpm/50 min, for [^{3}H]leukotriene B_4, [^{3}H]prostanoids and [^{3}H]arachidonic acid, respectively ($n = 6$). For comparison the total cellular radioactivity determined at the end of the incubation experiments amounted to 6 200 610 ± 446 276 dpm. The spontaneous rate of release declined by about 20–30% during the second incubation period. The Ca^{2+}

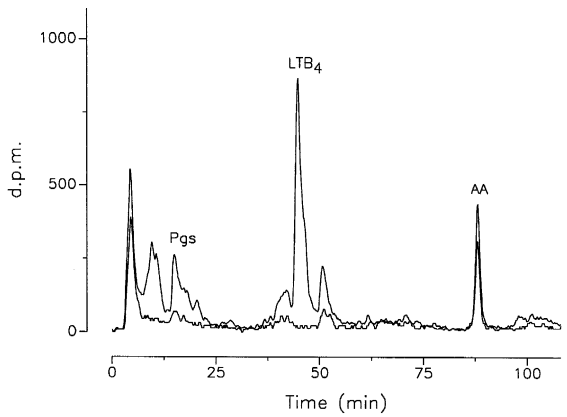

Fig. 1. Examples of original radiochromatograms of incubation media of freshly prepared rat alveolar macrophages, labelled with [^{3}H]arachidonic acid. Superimposed are two chromatograms of samples collected during two subsequent 50-min incubation periods, with A 23187 (10 μM) present during the second period. The separation of the ^{3}H-labelled compounds was achieved by reverse-phase gradient HPLC (Pgs, prostanoids; LTB_4, leukotriene B_4, AA, arachidonic acid; for further details see Section 2 and Fig. 2).

ionophore, A 23187 (10 μM), caused an increase in the release of all three fractions. In confirmation of previous observations (Reimann et al., 1995), the effect on the release of [^{3}H]leukotriene B_4, an increase by about 6500%, was by far the most pronounced (Fig. 2). The release of [^{3}H]prostanoids was increased by 550% (Fig. 2), whereas that of [^{3}H]arachidonic acid was increased by 215 ± 60% ($n = 6$; data not shown). Control experiments confirmed that the spontaneous and the evoked release of [^{3}H]prostanoids were almost completely suppressed by 100 μM acetylsalicylic acid and that of [^{3}H]leukotriene B_4 by 30 μM ICI 207698, an inhibitor of 5-lipoxygenase (Foster et al., 1990) (each $n = 2$, data not shown).

When the alveolar macrophages had been cultured for 18 h prior to the labelling with [^{3}H]arachidonic acid the spontaneous rate of release of [^{3}H]leukotriene B_4 and [^{3}H]prostanoids in the absence of any additional treatment

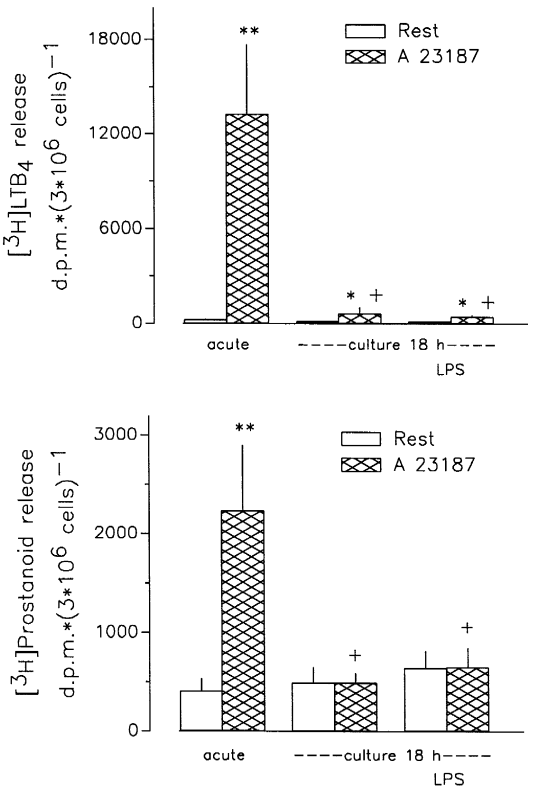

Fig. 2. Effects of an 18-h culture period, in the absence or presence of lipopolysaccharides (LPS, 10 μg/ml), on the subsequent release of [^{3}H]leukotriene B_4 ([^{3}H]LTB_4) (upper part) and [^{3}H]prostanoids (lower part) from rat alveolar macrophages, labelled with [^{3}H]arachidonic acid. Alveolar macrophages, either freshly prepared (acute) or after 18-h culture, were incubated for 2 h in Krebs medium containing 370 kBq [^{3}H]arachidonic acid (approx. 30 nM). Thereafter the release of ^{3}H-labelled compounds was determined in two subsequent 50-min periods, with A 23187 (10 μM) present during the second period. The release in the first period is given by open columns, that in the second period by shaded columns. Height of columns: radioactivity in the respective HPLC peaks (see Fig. 1), expressed as dpm per 3×10^6 cells, means ± S.E.M. of 5–8 experiments. Significance of differences: * $P < 0.05$, ** $P < 0.01$ from the respective resting period; $^+$ $P < 0.01$ from the corresponding value from acute experiments.

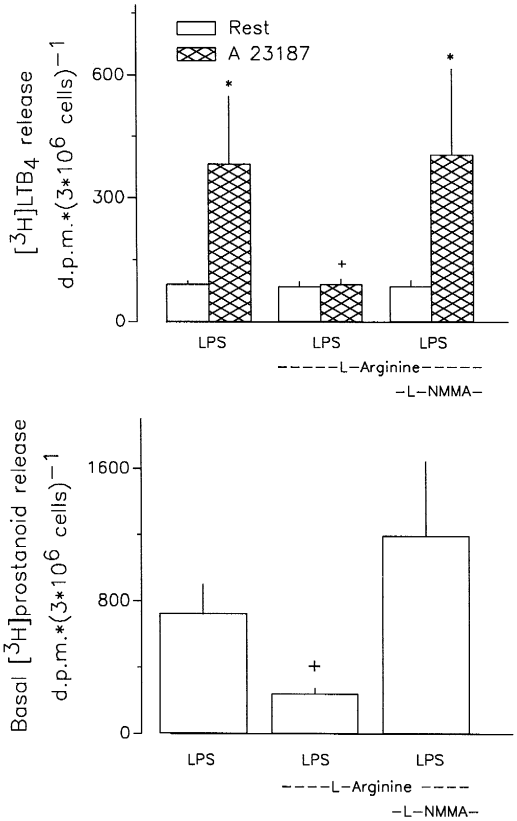

Fig. 3. Effects of L-arginine (300 μM) alone or in the presence of L-NMMA (100 μM) on the release of [^{3}H]leukotriene B_4 ([^{3}H]LTB_4) (upper part) and [^{3}H]prostanoids (lower part) from rat alveolar macrophages, labelled with [^{3}H]arachidonic acid. Alveolar macrophages were cultured for 18 h in the presence of 10 μg/ml lipopolysaccharides. Then they were labelled with [^{3}H]arachidonic acid and the release of ^{3}H-labelled compounds was determined as described in the legend to Fig. 2 (two incubation periods and A 23187 present during the second). The release in the first period is given by open columns, that in the second period by shaded columns (for prostanoids only the first incubation period is shown). In the respective experiments L-arginine and L-NMMA were added after the labelling period. Height of columns: radioactivity in the respective HPLC peaks (see Fig. 1), expressed as dpm per 3×10^6 cells, means ± S.E.M. of 5–8 experiments. Significance of differences: * $P < 0.05$, from the respective resting period; $^+$ $P < 0.05$ from the corresponding value without L-arginine.

was not affected (Fig. 2) whereas that of intact [^{3}H]arachidonic acid was significantly higher (17 881 ± 5199 dpm/50 min, $n = 6$; $P < 0.01$). On the other hand, the total cellular radioactivity (6 074 148 ± 342 575 dpm) was not different from that observed in freshly prepared cells. Most strikingly, in cultured alveolar macrophages, the effect of A 23187 on the release of [^{3}H]leukotriene B_4 was markedly reduced, although there was still a significant increase, by more than 500% (Figs. 2 and 3). In cultured alveolar macrophages, A 23187 failed, however, to evoke any significant increase in the release of [^{3}H]prostanoids (Fig. 2) or [^{3}H]arachidonic acid (not shown). The addition of lipopolysaccharides (10 μg/ml) to the culture medium had no significant effect on the subsequently measured spontaneous or evoked release of

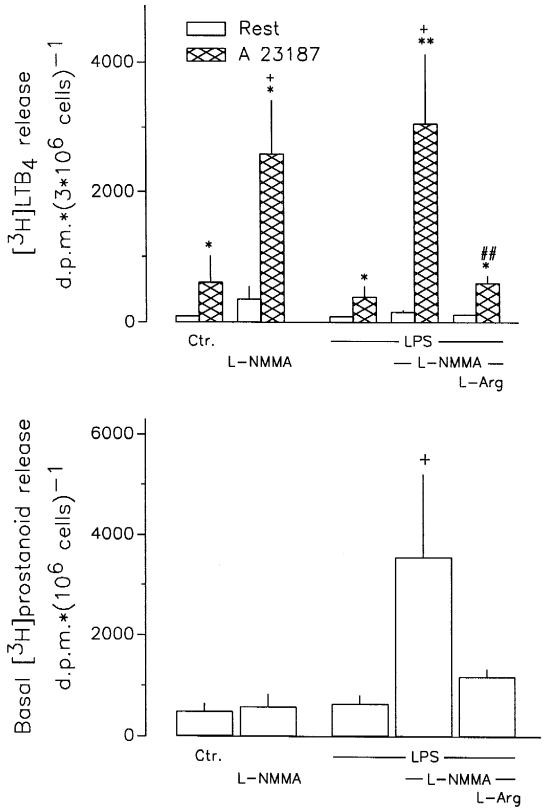

Fig. 4. Effects of culturing rat alveolar macrophages in the presence of L-NMMA alone or after elevation of L-arginine on subsequent release of [^{3}H]leukotriene B_4 ([^{3}H]LTB_4) (upper part) and [^{3}H]prostanoids (lower part). Alveolar macrophages were cultured for 18 h in the absence or presence of 10 μg/ml lipopolysaccharides (LPS) and/or 100 μM L-NMMA as indicated below the columns. Then they were labelled with [^{3}H]arachidonic acid and the release of ^{3}H-labelled compounds was determined as described in the legend to Fig. 2 (two incubation periods and A 23187 present during the second). The release in the first period is given by open columns and that in the second period by shaded columns (for prostanoids only the first incubation period is shown). In the respective experiments the L-arginine concentration of the culture medium was enhanced from 0.7 to 3 mM. Height of columns: radioactivity in the respective HPLC peaks (see Fig. 1), expressed as dpm per 3×10^6 cells, means ± S.E.M. of 5–8 experiments. Significance of differences: * $P < 0.05$, ** $P < 0.01$ from the respective resting period; $^{+}$ $P < 0.01$ from the corresponding value in the absence of L-NMMA; $^{\#\#}$ $P < 0.05$ from the corresponding value in the presence of L-NMMA alone.

[^{3}H]prostanoids, [^{3}H]leukotriene B_4 (Figs. 2 and 4) or [^{3}H]arachidonic acid (not shown), although the evoked release of [^{3}H]leukotriene B_4 tended to be reduced (Fig. 4). Since under all conditions tested in the present study, A 23187 failed to induce an increase in the release of [^{3}H]prostanoids from cultured alveolar macrophages, only the spontaneous [^{3}H]prostanoids release will be shown in the sections following.

3.2. Effects of endogenous NO generation on release of [^{3}H]arachidonic acid metabolites

When L-arginine (300 μM), the substrate of NO synthase, was added to the Krebs medium during the 'release period' of alveolar macrophages which had been cultured in the presence of lipopolysaccharides (i.e., a treatment shown to cause marked induction of NO synthase (Hey et al., 1995)), there was complete suppression of the evoked release of [^{3}H]leukotriene B_4 (Fig. 3). The addition of L-arginine also caused a significant reduction of the spontaneous release of [^{3}H]prostanoids (Fig. 3). Both effects of L-arginine were reversed when the NO synthase inhibitor, L-NMMA, was additionally present (Fig. 3). In a separate series of experiments (with separate controls), in which L-NMMA alone was added to the Krebs medium only during the 'release period', the A 23187-evoked release of [^{3}H]leukotriene B_4 and the spontaneous release of [^{3}H]prostanoids were not affected (each $n = 3$, data not shown).

In contrast to the Krebs medium, the culture medium normally contains L-arginine (700 μM). Therefore, in order to demonstrate putative effects of endogenous NO generated during the culture period, NO synthase was inhibited by the addition of 100 μM L-NMMA to the culture medium. Inhibition of NO synthase during the culture period (both in the absence and presence of lipopolysaccharides) resulted in a marked increase in the subsequent evoked release of [^{3}H]leukotriene B_4 (Fig. 4). The absolute levels of [^{3}H]leukotriene B_4 were similar, independent of whether the alveolar macrophages had been cultured in the presence of L-NMMA alone or together with lipopolysaccharides. However, the relative increase caused by L-NMMA in the presence of lipopolysaccharides was somewhat greater (about 700 vs. 300%) as lipopolysaccharides alone tended to reduce [^{3}H]leukotriene B_4 release.

Inhibition of NO synthase during the culture period also resulted in an enhanced [^{3}H]prostanoid release, but only in the experiments in which lipopolysaccharides were additionally present (Fig. 4).

Finally, the effects of L-NMMA on the evoked release of [^{3}H]leukotriene B_4 and that on the spontaneous [^{3}H]prostanoid release were reversed when the L-arginine concentration in the culture medium was increased from 0.7 to 3 mM (Fig. 4). In a separate series of experiments, D-arginine (in the corresponding concentration of 2.3 mM) added to the culture medium, in addition to lipopolysaccharides and L-NMMA, had no effect on the evoked release of [^{3}H]leukotriene B_4 and the spontaneous [^{3}H]prostanoid release (compared to experiments in which the culture medium contained only lipopolysaccharides and L-NMMA alone, each $n = 4$, data not shown).

4. Discussion

The present experiments showed that the in vitro release of arachidonic acid-derived mediators, both of the cyclooxygenase and lipoxygenase pathway, from rat alveolar macrophages can be inhibited by endogenous NO. The

results indicated further that endogenous NO may inhibit the activity as well as the availability (expression or stability) of both enzymes in rat alveolar macrophages.

In the present study a radiotracer technique was used to measure the release of arachidonic acid-derived metabolites. Alveolar macrophages, either freshly prepared or after an 18-h culture period, were labelled with [^{3}H]arachidonic acid and subsequently the release of ^{3}H-labelled compounds was determined. [^{3}H]Arachidonic acid metabolites were separated by reverse-phase HPLC using a gradient system which allows the separation of all major eicosanoids (arachidonic acid, leukotrienes, different prostaglandins, and several hydroxyeicosatetraenoic acid (HETE) fractions, (Henke et al., 1984; Van Scott et al., 1990; Brunn et al., 1995b). Although some prostanoids were only insufficiently separated, this gradient system appeared to be suitable for the purposes of the present study as a clear separation of intact arachidonic acid, the metabolites of the cyclo-oxygenase pathway and those of the lipoxygenase pathway was achieved within one chromatogram (see Fig. 1).

With freshly prepared alveolar macrophages, the Ca^{2+} ionophore, A 23187, stimulated the release of [^{3}H]leukotriene B_4 much more than that of [^{3}H]prostanoids (a 65-fold vs. 5- to 6-fold increase, respectively). Differential activation by the Ca^{2+} ionophore of the lipoxygenase and cyclo-oxygenase pathway in rats had been observed in a previous study in which it was additionally shown that activation of protein kinase C by phorbol 12-myristate 13-acetate caused a selective increase in the release of prostanoids (Reimann et al., 1995).

After 18 h in culture the stimulatory effect of A 23187 on the release of [^{3}H]leukotriene B_4 was largely reduced and that on the release of [^{3}H]prostanoids was abolished. It is unclear whether the high responsiveness of the freshly prepared alveolar macrophages reflects the 'normal' in vivo responsiveness or whether the isolation procedure caused some kind of 'priming', an effect lost again during the culture period. Addition of lipopolysaccharides to the culture medium, a treatment which causes a very large induction of NO synthase in these cells (Hey et al., 1995; Hecker et al., 1995), had no significant effect on the subsequent release of [^{3}H]leukotriene B_4 and [^{3}H]prostanoids. It should, however, be noted that amino acid-free Krebs medium was used during the 'release period'. Therefore, significant amounts of endogenous NO might not have been produced under these conditions because the substrate of inducible NO synthase, L-arginine, was lacking. When L-arginine was added to the incubation medium of alveolar macrophages which had been cultured in the presence of lipopolysaccharides (i.e., inducible NO synthase was markedly induced) there was complete suppression of the evoked [^{3}H]leukotriene B_4 release and marked reduction of the spontaneous release of [^{3}H]prostanoids, indicating that the acute generation of endogenous NO can inhibit both the lipoxygenase pathway and the cyclo-oxygenase pathway in alveolar macrophages. The fact that the observed inhibitory effects of L-arginine were reversed by the NO synthase inhibitor, L-NMMA, further supports the conclusion that indeed NO was mediating these effects, although other NO-derived reactive molecules, such as peroxynitrite or dinitrosyl-iron dithiolate (Vanin et al., 1993) could also be involved.

The synthesis of [^{3}H]leukotriene B_4 involves a cascade of enzymes (phospholipase A_2–5-lipoxygenase activating protein (FLAP)–5-lipoxygenase and the leukotriene A synthase and hydrolase) (see Steinhilber, 1994) and the present experiments do not allow a final conclusion about the target enzyme(s) of NO. However, 5-lipoxygenase is an iron-containing enzyme and it has been shown that NO can directly inhibit the activity of this enzyme, probably by reducing the protein-bound iron and/or interfering with the iron binding site of the enzyme (Kanner et al., 1992). Similarly, cyclo-oxygenase, the key enzyme in prostaglandin synthesis, is an iron-containing heme protein and NO has been shown to interact with this enzyme also. However, the effects of NO on cyclo-oxygenase appear to be more complex, as cyclo-oxygenase activity can be enhanced by NO in low concentrations, but is inhibited by NO in high concentrations (Kanner et al., 1992; Salvemini et al., 1993; Swierkosz et al., 1995). The amounts of NO formed in the present experiments (after induction of inducible NO synthase by lipopolysaccharides) appear to have been sufficiently high to inhibit markedly cyclo-oxygenase.

In addition to its effects on the acute enzyme activities, NO also appears to inhibit the availability of the enzymes of the cyclo-oxygenase and lipoxygenase pathway. When NO synthesis was inhibited by L-NMMA during the culture period, the subsequently determined release of [^{3}H]leukotriene B_4 evoked by A 23187 was markedly enhanced, suggesting that the above described reduction in the responsiveness of the lipoxygenase pathway after the culture period may in part be caused by endogenous NO. Interestingly, the evoked release of [^{3}H]leukotriene B_4 was very similar when L-NMMA was present alone or in combination with lipopolysaccharides, indicating that even in the absence of lipopolysaccharides functionally significant amounts of NO might have been produced. Indeed, rat alveolar macrophages cultured under the same conditions as in the present study showed a significant NO synthase activity even in the absence of lipopolysaccharides (determined from the accumulation of nitrite as well as the conversion of [^{3}H]L-arginine into [^{3}H]L-citrulline) (Hey et al., 1995; Hecker et al., 1995). The suggestion that the effect of L-NMMA was mediated via specific inhibition of NO synthase is supported by the observation that it was counteracted by increasing the L-arginine concentration in the culture medium. Whether NO decreased the stability of the enzymes of the lipoxygenase pathway (possibly that of 5-lipoxygenase) or whether NO inhibited their de novo synthesis cannot be decided from the present results. In

HL60 cells, exposed to dimethyl sulfoxide (DMSO) in order to differentiate them to mature granulocyte-like cells, it was observed that a pool of 5-lipoxygenase accumulated during maturation and this pool was then 'consumed' depending on the activation state of the cells (Kargman and Rouzer, 1989). Whether similar mechanisms may also apply for alveolar macrophages is unknown. The present observations at least, indicate that lipopolysaccharides may not act as inductors of the lipoxygenase pathway in rat alveolar macrophages.

Somewhat different mechanisms appear to operate with regard to the cyclo-oxygenase pathway. In J774.2 macrophages, it has been shown that the induction of cyclo-oxygenase-2 by lipopolysaccharides is inhibited by the co-induction of inducible NO synthase (Swierkosz et al., 1995), and similar interactions may occur in rat alveolar macrophages. In support of this possibility are the present observations that an enhanced [^{3}H]prostanoid release occurred only when lipopolysaccharides had been present during the culture period in combination with L-NMMA, whereas the presence of either L-NMMA alone or lipopolysaccharides alone had no significant effect.

The reasons for the enhanced free arachidonic acid in alveolar macrophages after the culture period are unclear at present. Enhanced activity of phospholipase A_2 might be involved. However, under these conditions, cyclo-oxygenase activity appeared to be rate limiting for the generation of prostanoids, whereas in freshly prepared alveolar macrophages the availability of free arachidonic acid appeared to be rate limiting. This is indicated by the observation that A 23187 enhanced [^{3}H]prostanoid release in freshly prepared alveolar macrophages, but did not cause any increase in [^{3}H]prostanoid release from alveolar macrophages which had been cultured under the various conditions. On the other hand, 'induction' of cyclo-oxygenase (by culture in the presence of lipopolysaccharides and L-NMMA) resulted in an increase in the subsequent release of [^{3}H]prostanoids without any additional stimulus.

In conclusion, the present experiments showed that, in rat alveolar macrophages, NO generated by inducible NO synthase can inhibit the release of arachidonic acid-derived mediators, both of the cyclo-oxygenase and lipoxygenase pathway, through multiple sites of action. The inhibition of leukotriene release could be of particular significance as a potential anti-inflammatory and possibly also as an anti-broncho-obstructive effect. In this context it should be mentioned that in the guinea pig, different NO synthase inhibitors cause hyperresponsiveness of the airways towards various stimuli (Nijkamp et al., 1993) and an augmentation of antigen-induced broncho-constriction (Persson et al., 1993). Furthermore, very recently it was reported that the airway hyperresponsiveness following NO synthase inhibition could be inhibited by a 5-lipoxygenase inhibitor or a non-selective leukotriene receptor antagonist (Folkerts et al., 1995). Thus, returning to the discussion about the importance of inducible NO synthase in inflammatory airway diseases (see Section 1), these observations together with the present results suggest that NO generated by inducible NO synthase could have also an anti-inflammatory and anti-obstructive potential.

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