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The inhibitory effects of mercaptoalkylguanidines on cyclo-oxygenase activity

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1 It has been proposed that in inflammatory conditions, in which both the inducible isoforms of nitric oxide synthase (iNOS) and cyclo-oxygenase (COX-2) are induced, inhibition of NOS also results in inhibition of arachidonic acid metabolism. In the present study we have investigated whether mercaptoalkylguanidines, a novel class of selective iNOS inhibitors, may also influence the activity of cyclo-oxygenase (COX). Therefore, the effect of mercaptoethylguanidine (MEG) and related compounds on the activity of the constitutive (COX-1) and the inducible COX (COX-2) was investigated in cells and in purified enzymes. Aminoguanidine, N^G-methyl-L-arginine (L-NMA) and N^G-nitro-L-arginine methyl ester (L-NAME) were also studied for comparative purposes.

2 Western blot analysis demonstrated a significant COX-1 activity in unstimulated J774 macrophages and in unstimulated human umbilical vein endothelial cells (HUVEC). Immunostimulation of the J774 macrophages by endotoxin (lipopolysaccharide of *E. coli*, LPS 10 μg ml^{-1}) and interferon γ (IFNγ, 100 u ml^{-1}) for 6 h resulted in a significant induction of COX-2, and a down-regulation of COX-1. No COX-2 immunoreactivity was detected in unstimulated HUVEC or unstimulated J774 cells. Therefore, in subsequent studies, the effect of mercaptoalkylguanidines on COX-1 activity was studied in HUVEC stimulated with arachidonic acid for 6 h, and in J774 cells stimulated with arachidonic acid for 30 min. The effect of mercaptoalkylguanidines on COX-2 activity was studied in immunostimulated J774 macrophages, both on prostaglandin production by endogenous sources, and on prostaglandin production in response to exogenous arachidonic acid stimulation. In addition, the effect of mercaptoalkylguanidines on purified COX-1 and COX-2 activities was also studied.

3 In experiments designed to measure COX-1 activity in HUVEC, the cells were stimulated by arachidonic acid (15 μM) for 6 h. This treatment induced a significant production of 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$, the stable metabolite of prostacyclin), while nitrite production was undetectable by the Griess reaction. MEG (1 μM to 3 mM) caused a dose-dependent inhibition of the accumulation of 6-keto-$PGF_{1\alpha}$, with an IC_{50} of 20 μM. However, aminoguanidine, L-NAME or L-NMA (up to 3 mM) did not affect the production of 6-keto-$PGF_{1\alpha}$ in this experimental system. In experiments designed to measure COX-1 activity in J774.2 macrophages, the cells were stimulated by arachidonic acid (15 μM) for 30 min; this also induced a significant production of 6-keto-$PGF_{1\alpha}$ and MEG (1 μM to 3 mM), aminoguanidine (at 1 and 3 mM), but neither L-NAME nor L-NMA inhibited the production of prostaglandins.

4 In experiments designed to measure prostaglandin production by COX-2 with endogenous arachidonic acid, J774.2 cells were immunostimulated for 6 h in the absence or presence of various inhibitors. In experiments designed to measure prostaglandin production by COX-2 with exogenous arachidonic acid, J774.2 cells were immunostimulated for 6 h, followed by a replacement of the culture medium with fresh medium containing arachidonic acid and various inhibitors. Both of these treatments induced a significant production of 6-keto-$PGF_{1\alpha}$. Nitrite production, an indicator of NOS activity, was moderately increased after immunostimulation. MEG (1 μM to 3 mM) caused a dose-dependent inhibition of the accumulation of COX metabolites. Similar inhibition of LPS-stimulated 6-keto $PGF_{1\alpha}$ production was shown by other mercaptoalkylguanidines (such as N-methyl-mercaptoethylguanidine, N,N′-dimethyl-mercaptoethylguanidine, S-methyl-mercaptoethylguanidine and guanidino-ethyldisulphide), with IC_{50} values ranging between 34–55 μM. However, aminoguanidine, L-NAME and L-NMA (up to 3 mM) did not affect the production of prostaglandins.

5 In comparative experiments indomethacin, a non selective COX inhibitor, and NS-398, a selective COX-2 inhibitor, reduced (LPS) stimulated 6-keto-$PGF_{1\alpha}$ production in J774 macrophages in a dose-dependent manner without affecting nitrite release. Indomethacin, but not NS-398, inhibited 6-keto-$PGF_{1\alpha}$ production in the HUVECs.

6 The inhibitory effect of MEG was due to direct inhibition of the catalytic activity of COX as indicated in experiments with purified COX-1 and COX-2. MEG dose-dependently inhibited the purified COX-1 and COX-2 activity with IC_{50} values of 33 μM and 36 μM, respectively. Aminoguanidine (at the highest concentrations) inhibited the formation of COX-1 metabolites, without affecting COX-2 activity. High doses of L-NAME (3 mM) decreased COX-1 activity only, while L-NMA (up to 3 mM) had no effect on the activity of either enzyme.

7 These results suggest that MEG and related compounds are direct inhibitors of the constitutive and the inducible cyclo-oxygenases, in addition to their effects on the inducible NOS. The additional effect of mercaptoalkylguanidines on COX activity may contribute to the beneficial effects of these agents in inflammatory conditions where both iNOS and COX-2 are expressed.

Keywords: Prostaglandins nitric oxide; inflammation; shock; mercaptoalkylguanidines; endotoxin

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Introduction

Prostaglandins, produced by cyclo-oxygenases (COX), and nitric oxide (NO), produced by NO synthases (NOS), are important biological mediators. The COX and NOS pathways share similarities (see Smith *et al*., 1991 and Moncada *et al*., 1991 for reviews). The activities of both enzymes are dependent upon haeme as a cofactor (Smith *et al*., 1991; Marletta, 1993). In addition, both enzymes are present in constitutive and inducible isoforms. The constitutively expressed isoforms of COX and NOS release prostaglandins and NO, respectively, serving physiological purposes in various organs of the body.

During the process of host defence, as in inflammation and shock, endotoxins and cytokines induce rapid alterations in cellular immediate-early gene expression leading to the *de novo* synthesis of the inducible COX (COX-2) (Maier *et al*., 1990; Vane *et al*., 1994; and for review see Mitchell *et al*., 1995) and the inducible NOS (iNOS) pathways (see Moncada *et al*., 1991; Szabó, 1995 for review), with subsequent enhanced formation of prostaglandins and NO. Co-induction of iNOS and COX-2 has been shown in several cell types, including murine macrophages (Salvemini *et al*., 1993; Akarasereenont *et al*., 1994; Swierkosz *et al*., 1995), smooth muscle cells (Inoue *et al*., 1993), pancreatic islet cells (Corbett *et al*., 1993), with similarities in the signal transduction pathways.

It is now evident that the NOS and COX pathways are interrelated and the cross-talk between these two pathways is important in the regulation of the inflammatory process. Endogenous NO has been demonstrated to increase prostaglandin biosynthesis in *in vivo* and *ex vivo* models of inflammation and endotoxic shock, whereas inhibition of NO synthesis by NOS inhibitors results in reduction of prostaglandin production *in vivo* and *in vitro* (Sautebin & Di Rosa, 1994; Sautebin *et al*., 1995; Salvemini *et al*., 1993; 1994; 1995).

Recently, major efforts have been focused on identifying anti-inflammatory drugs which can inhibit the catalytic activity of NOS and COX, preferentially with selectivity towards the inducible isoforms. Pharmacological agents that can suppress the expression of iNOS and COX-2 (such as tyrosine kinase inhibitors and glucocorticoids) have also been described (Smith *et al*., 1991; Moncada *et al*., 1991; Mitchell *et al*., 1995).

Recently, S-substituted isothioureas have been described as a novel class of potent NOS inhibitors (Szabó *et al*., 1994; Garvey *et al*., 1994; Southan *et al*., 1995). In contrast to the most commonly used non isoform selective NOS inhibitors, such as the L-arginine analogues N^G-methyl-L-arginine (L-NMA) and N^G-nitro-L-arginine-methyl ester (L-NAME), aminoalkylisothioureas are S-substituted isothioureas, are not related to L-arginine and show selectivity towards iNOS. S-substituted isothioureas have been shown to exert beneficial effects in endotoxic shock (Szabó *et al*., 1994; Garvey *et al*., 1994; Thiemermann *et al*., 1995; Southan *et al*., 1996; Southan & Szabó, 1996). For instance, aminoethyl-isothiourea (AE-TU) has been shown to improve survival, haemodynamic status, vascular contractility and organ function in rodent models of endotoxic shock (Szabó *et al*., 1995; Thiemermann *et al*., 1995). In *in vivo* and *in vitro* studies, the pharmacological actions of aminoalkylisothioureas are due to their rearrangement to mercaptoalkylguanidines, which represent a novel class of NOS inhibitors, with selectivity towards iNOS (Southan *et al*., 1996; Szabó *et al*., 1996; Southan & Szabó, 1996). For instance, AE-TU rearranges to form mercaptoethylguanidine (MEG), an inhibitor of iNOS activity (Southan *et al*., 1996).

The present study was designed to characterize whether mercaptoalkylguanidines also modulate the synthesis of proinflammatory prostaglandins. We have investigated (i) whether treatment with MEG and related compounds affects the synthesis of prostacyclin and thromboxane A_2 (TxA_2) in arachidonic acid-stimulated human umbilical vein endothelial cells (HUVEC) and in immunostimulated J774.2 macrophages; (ii) whether the effect of these drugs on COX activity is secondary to inhibition of NOS; and (iii) whether MEG and related compounds have direct effect on the activities of purified COX-1 and COX-2. Comparative experiments were also carried out to investigate the effects of the L-arginine analogue NOS inhibitors L-NMA and L-NAME (see Moncada *et al*., 1991 for review), and the guanidine iNOS inhibitor aminoguanidine (Hasan *et al*., 1993) on COX activity.

Methods

Cell culture

Murine macrophages J774.2 were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal calf serum and 4 mM L-glutamine in 96 well plates (200 μl final volume) under standard conditions. Human umbilical vein endothelial cells (HUVEC) were cultured in F12K nutrient medium containing 10% foetal calf serum, heparin (100 $\mu g\ ml^{-1}$) and endothelial cell growth supplement (30 $\mu g\ ml^{-1}$) in 96 well plates (200 μl final volume) under standard conditions. Cells were used at 90–100% confluence. Immediately before the experiments, culture medium was replaced by fresh medium without foetal calf serum in order to avoid interference with radioimmunoassay.

COX-1 and COX-2 Western blotting

HUVECs or J774 cells were cultured to 90% confluency. J774 cells were treated with vehicle or with endotoxin of *E. coli* (10 $\mu g\ ml^{-1}$) and interferon γ (IFNγ, 100 u ml^{-1}) for 6 h. Cells were then washed with cold phosphate-buffered saline (PBS), scraped into cold PBS and transferred into microfuge tubes. After centrifugation, PBS was removed and cells were resuspended in RIPA buffer consisting of PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS), 10 $\mu g\ ml^{-1}$ aprotinin and 0.5 mM phenylmethylsulphonylfluoride (PMSF). DNA was sheared by repeated passage through a 22 gauge needle. Cells were incubated on ice for 30 min, then centrifuged at 14 000 *g* for 30 min at 4°C.

Sixty micrograms of each cell homogenate or authentic COX-1 or COX-2 standards were diluted in SDS treatment buffer and heated to 95°C for 3 min. Proteins were then loaded onto a gradient gel (8–16% Tris-glycine) and run at 125 V for 2 h, then transferred to 0.45 μm nitrocellulose by wet electroblot with a 1/2xTowbin buffer.

Membranes were blocked with 1% BSA in PBS for 1 h, then probed with rabbit anti- COX-1 (1 : 1000) or anti- COX-2 in 1% BSA in PBS-T (0.025% Tween 20) for 2 h at room temperature. The blot was then washed twice with PBS-T for 5 min, once for 15 min and incubated for 1 h with a secondary antibody, goat anti-rabbit IgG-AP (1 : 3000). The blot was washed twice with PBS-T then with PBS, followed by colour development with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate in Tris: $MgCl_2$ buffer for 20 min. Using authentic COX-1 and COX-2 enzymes, we have confirmed the isoform specificity and the lack of detectable isoform cross-reactivity of the COX antibodies used in the present study (not shown).

Activation of COX-1 in HUVECs

Cells were pretreated for 30 min with MEG, L-NMA, L-NAME or aminoguanidine (1 μM–3 mM). After 30 min of incubation cells were stimulated with arachidonic acid (15 μM) in order to activate the constitutive COX-1. Cells were then incubated for a further period of 6 h and the supernatant collected for measurement of arachidonic acid metabolism by

radioimmunoassay. In a different set of experiments, cells were pretreated for 30 min with indomethacin or NS-398 (1 nM–10 μM).

Activation of COX-1 in J774 cells

Cells were pretreated with MEG, other mercaptoalkylguanidines, L-NMA, L-NAME or aminoguanidine (1 μM–3 mM) for 30 min; they were then stimulated with arachidonic acid (15 μM) in order to activate the constitutive COX, incubated for a further period of 30 min and the supernatant collected for the measurement of arachidonic acid metabolism by radioimmunoassay.

Activation of COX-2 in J774 cells: endogenous substrate

Cells were pretreated with MEG, other mercaptoalkylguanidines, L-NMA, L-NAME or aminoguanidine (1 μM–3 mM) for 30 min. In a different set of experiments, cells were pretreated for 30 min with indomethacin or NS-398 (1 nM–10 μM). J774 cells were then stimulated with endotoxin of *E. coli* lipopolysaccharide (LPS, 10 μg ml^{-1}) and interferon γ (IFNγ, 100 u ml^{-1}) in order to induce COX-2. They were then incubated for a further period of 6 h and the supernatant collected for the measurement of arachidonic acid metabolism by radioimmunoassay.

Activation of COX-2 in J774 cells: exogenous substrate

J774 cells were stimulated with endotoxin of *E. coli* (LPS, 10 μg ml^{-1}) and interferon γ (IFNγ, 100 u ml^{-1}) for 6 h in order to induce COX-2. The supernatant of the cells was replaced with fresh medium containing arachidonic acid (15 μM) in the absence or presence of MEG and other inhibitors. Cells were then incubated for a further period of 30 min and the supernatant collected for the measurement of arachidonic acid metabolism by radioimmunoassay.

Analysis of 6-keto-PGF$_{1\alpha}$ and thromboxane B$_2$ by radioimmunoassay

Supernatant samples were diluted 1 : 5 in a buffer containing 0.1% polyvinylpyrrolidone, 0.9% NaCl, 50 mM Tris base, 1.7 mM $MgSO_4$ and 0.16 mM $CaCl_2$ (pH 7.4) before radioimmunoassay. The stable metabolites of prostacyclin, 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-PGF$_{1\alpha}$), and of thromboxane A_2 and thromboxane B_2 (TxB_2), were determined by radioimmunoassay as described by Wise *et al*. (1983).

Nitrite production

Nitrite production, an indicator of NO synthesis, was measured in the supernatant of J774.2 macrophages by using the spectrophotometric Griess reaction (Green *et al*., 1982). Aliquots of supernatant (100 μl) were mixed with 100 μl of Griess reagent (1% sulphanilamide and 0.1% naphthyl ethylenediamine in 5% phosphoric acid). The optical density at 550 nm (OD_{550}) was measured by using the Spectramax microplate reader. Nitrite concentrations were calculated by comparison with OD_{550} of standard solutions of sodium nitrite prepared in culture medium.

Cell viability

Mitochondrial respiration, an indicator of cell viability, was assessed by the mitochondrial-dependent reduction of 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) to formazan (Southan *et al*., 1996). At the end of each experiment, cells in 96-well plates were incubated with MTT (0.2 mg ml^{-1}) for 1 h at 37°C. The culture medium was removed and the cells were solubilized in dimethylsulphoxide (100 μl). The extent of reduction of MTT to formazan within cells was quantitated by measurement of optical density at 550 nm by using the Spectramax microplate reader.

Experiments on purified COX-1 and COX-2

In a test tube containing 0.1 M Tris-HCL buffer (pH 8.0, 2 ml final volume) with 1 mM EDTA, 0.2 mM phenol and 1 μM hemin, 10 units of COX-1 or COX-2 were allowed to react with 100 μM arachidonic acid for two minutes at 37°C in the presence or absence of MEG, L-NMA, L-NAME or aminoguanidine (1 μM–3 mM). The reaction was quenched by addition of 50 μl stannous chloride solution (100 mg ml^{-1} in 1 M HCl) and was allowed to proceed for an additional period of 10 min before it was stopped thereafter by addition of 5 ml of a buffer containing 0.1% polyvinylpyrrolidone, 0.9% NaCl, 50 mM Tris base, 1.7 mM $MgSO_4$ and 0.16 mM $CaCl_2$ (pH 7.4). Changes in COX activity were determined by measuring the accumulation of $PGF_{2\alpha}$, the stable metabolite of PGH_2, in reaction samples by using Enzyme immunoassay kits (Cayman Chemical, Ann Arbor, MI, U.S.A.).

Materials

Arachidonic acid, bacterial lipopolysaccharide (*E.coli*, serotype No. 0127:B8), indomethacin, MTT, N^G-nitro-L-arginine methyl ester (L-NAME) were obtained from Sigma (St. Louis, MO, U.S.A.). DMEM, F12K medium and foetal calf serum were from Gibco (Grand Island, NY, U.S.A.). N^G-methyl-L-arginine monoacetate (L-NMA) was obtained from Calbiochem (La Jolla, Ca, U.S.A.). Murine IFNγ was purchased by Genzyme (Cambridge, MA, U.S.A.). Purified COX-1 (ovine), COX-2 (ovine), polyclonal antibodies against COX-1 (rabbit, raised against a synthetic peptide from ovine COX-1) and COX-2 (rabbit, raised against a 17 amino acid peptide from murine sequence), and Enzyme immunoassay kits were obtained from Cayman Chemical (Ann Arbor, MI, U.S.A.). Aminoethylisothiourea and aminoguanidine were obtained from Aldrich (St. Louis, MO, U.S.A.). Mercaptoalkylguanidines were synthesized from their corresponding aminoalkylisothiourea precursors as previously described (Southan *et al*., 1996). NS-398 (N-(2-cyclohexyloxy-4-nitrophenyl) methane sulphonamide) was obtained from Biomol Research Laboratories (Plymouth Meeting, PA, U.S.A.). All other compounds and reagents were obtained from Sigma.

Statistical analysis

In each experimental day, triplicate wells were used for the various treatment conditions. All values in the figures are expressed as means $\pm$ s.e.mean of values obtained in 6–12 wells, collected from 2–4 independent experiments. Student's unpaired *t* test was used to compare means between groups. A *P* value less than 0.05 was considered statistically significant.

Results

Identification of COX isoforms in HUVECs and J774 cells

Antibodies against COX-1 recognized a band of approximately 70 kDa in the extracts of HUVEC and in the extracts of unstimulated J774 macrophages (Figure 1). Immunostimulation of the J774 macrophages by endotoxin (lipopolysaccharide of *E. coli*, LPS 10 μg ml^{-1}) and IFNγ (100 u ml^{-1}) for 6 h resulted in the appearance of a band of 70 kDa which was recognized by antibodies against COX-2 (Figure 1). We did not detect any COX-2 activity in the HUVECs and in unstimulated J774 cells (Figure 1). Moreover, we found a decrease in the intensity of the band

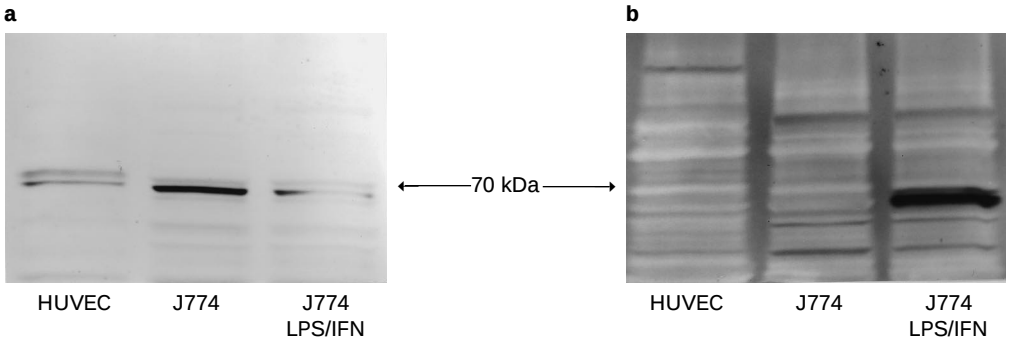

Figure 1 Western blots with polyclonal antibodies to cyclo-oxygenase-1 (COX-1; a) or COX-2 (b) in human umbilical vein endothelial cells (HUVECs), unstimulated J774 macrophages and in J774 macrophages exposed to LPS ($10\,\mu g\,ml^{-1}$) and IFNγ ($100\,u\,ml^{-1}$) for 6 h. Equal amounts of protein were loaded in all lanes. Similar results were obtained in 3 independent experiments.

recognized by COX-1 antibody following treatment of the J774 cells with LPS and IFNγ (Figure 1).

The presence of COX-1 and the lack of COX-2 in the HUVECs and in the unstimulated J774 cells suggested that these cells can serve as suitable models to test the effects of various inhibitors on COX-1 activity. Moreover, the presence of an intense band representing COX-2 in the J774 cells after immunostimulation suggested that immunostimulated J774 cells can serve as a model to test the effect of various agents on COX-2 activity.

Effects of mercaptoalkylguanidines on COX-1 activity in HUVECs

Stimulation with arachidonic acid (15 μM) induced a significant increase of 6-keto-$PGF_{1\alpha}$ (2.36 ± 0.14 ng ml^{-1}) levels in comparison to unstimulated control cells (1.44 ± 0.11 ng ml^{-1}, $P<0.001$). In the presence of increasing concentrations of MEG (from 1 μM up to 3 mM) a dose-dependent inhibition of 6-keto-$PGF_{1\alpha}$ and TxB_2 production was observed, with an IC_{50} of 20 μM (Figure 2). In contrast, aminoguanidine, L-NMA and L-NAME did not exert any inhibitory effect on the production of the arachidonic acid metabolites (Figure 2).

Nitrite production was not detectable in the HUVECs stimulated with arachidonic acid, with the Griess reaction (not shown). The observed inhibitory effects were not associated with reduction of cell viability, as mitochondrial respiration was not significantly altered at the drug concentrations studied (up to 3 mM) (not shown).

Effects of mercaptoalkylguanidines on COX-1 activity in J774.2 cells

Stimulation of J774 cells with arachidonic acid (15 μM) induced a significant increase of 6-keto-$PGF_{1\alpha}$ (4.1 ± 1.5 ng ml^{-1}) and TxB_2 (1.8 ± 0.6 ng ml^{-1}) levels in comparison to unstimulated control cells (0.9 ± 0.3 and 0.4 ± 0.3 ng ml^{-1}, respectively; $P<0.001$). In the presence of increasing concentrations of MEG (from 1 μM up to 3 mM) a dose-dependent inhibition of 6-keto-$PGF_{1\alpha}$ and TxB_2 production was observed (Figure 3).

Aminoguanidine partially reduced the production of arachidonic acid metabolites at the highest concentrations tested (1 and 3 mM, Figure 1). L-NMA and L-NAME did not exert any inhibitory effect on the production of the arachidonic acid metabolites (Figure 3).

Nitrite production was not detectable in the J774.2 macrophages stimulated with arachidonic acid (not shown). The observed inhibitory effects were not associated with reduction of cell viability, as mitochondrial respiration was not significantly altered at the drug concentrations studied (up to 3 mM) (not shown).

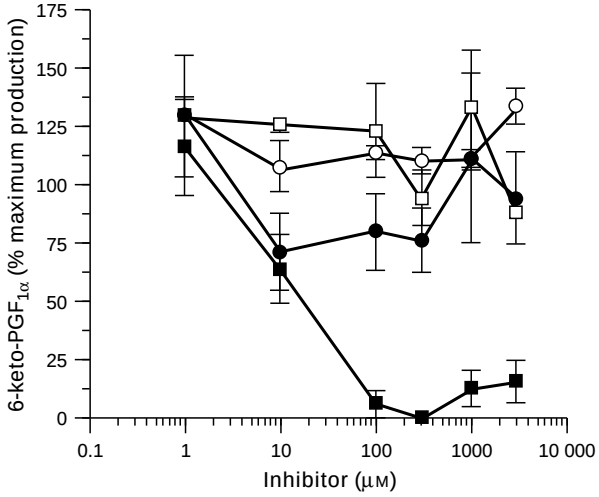

Figure 2 Effect of mercaptoethylguanidine (MEG; ■), aminoguanidine (□), L-NAME (○) and L-NMA (●) (each at 1 μM – 3 mM) on 6-keto-$PGF_{1\alpha}$ production in HUVECs exposed to arachidonic acid (15 μM) for 6 h. The means ± s.e.mean (vertical lines) for 6 determinations performed on 2 separate experimental days are shown. Cells were incubated for 30 min with the inhibitors before the addition of arachidonic acid.

Effects of mercaptoalkylguanidines on COX-2 activity in J774.2 cells: endogenous arachidonic acid stimulation

Stimulation with LPS ($10\,\mu g\,ml^{-1}$) and IFNγ ($100\,u\,ml^{-1}$) induced a significant increase of 6-keto-$PGF_{1\alpha}$ (4.3 ± 1.7 ng ml^{-1}) and TxB_2 (1.5 ± 0.3 ng ml^{-1}) levels in comparison to unstimulated control cells (0.9 ± 0.4 and 0.33 ± 0.03 ng ml^{-1}, respectively; $P<0.001$). In the presence of increasing concentrations of MEG (from 1 μM up to 3 mM) a dose-dependent inhibition of 6-keto-$PGF_{1\alpha}$ and TxB_2 production was observed (Figure 4). Substituted derivatives of mercaptoalkylguanidines exerted a similar dose-dependent inhibition on prostaglandin metabolism with IC_{50} values ranging between 33 and 55 μM (Table 1).

The inhibitory effect was characteristic of the mercaptoalkylguanidine group of NOS inhibitors, since NOS inhibitors with a different chemical structure did not affect prostaglandin production. Aminoguanidine, L-NMA and L-NAME did not exert any inhibitory effect on 6-keto-$PGF_{1\alpha}$ and TxB_2 production (Figure 5). Conversely, L-NMA, at the highest concentrations used (1 and 3 mM) significantly increased the release of 6-keto-$PGF_{1\alpha}$ and TxB_2 (Figure 5), in agreement with previous observations (Swierkosz *et al.*, 1995).

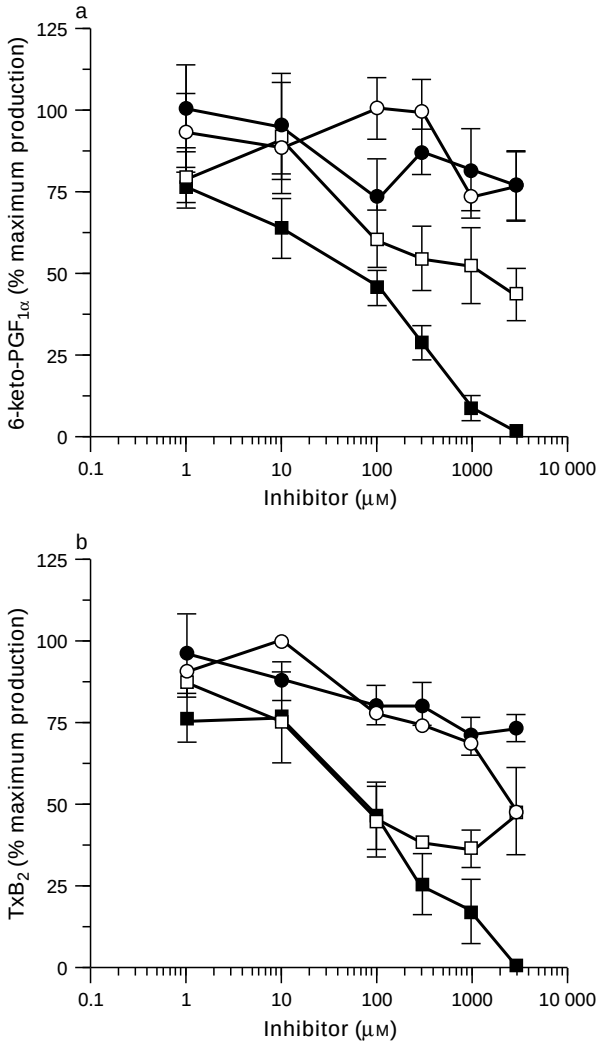

Figure 3 Effect of mercaptoethylguanidine (■), aminoguanidine (□), L-NAME (○) and L-NMA (●) (each at 1 μM – 3 mM) on 6-keto-$PGF_{1\alpha}$ (a) or TxB_2 (b) production in J774.2 macrophages exposed to arachidonic acid (15 μM) for 30 min. The means ± s.e.mean (vertical lines) are shown for 12 determinations performed on 4 separate experimental days. Cells were incubated for 30 min with the inhibitors before the addition of arachidonic acid.

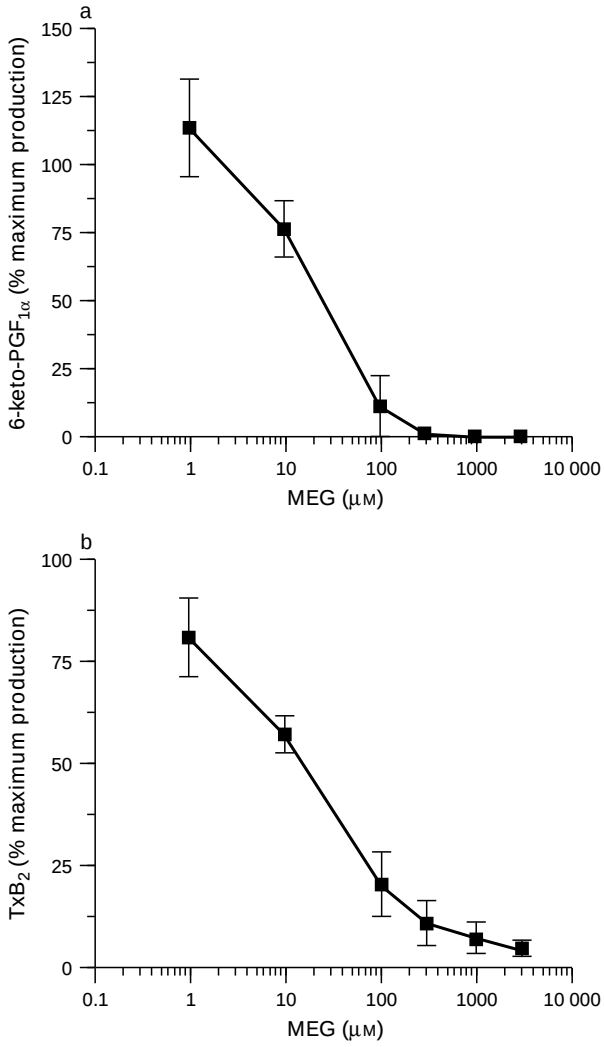

Figure 4 Dose-dependent inhibition by mercaptoethylguanidine (MEG; 1 μM – 3 mM) of 6-keto-$PGF_{1\alpha}$ (a) or TxB_2 (b) production in J774.2 macrophages exposed to LPS (10 μg ml^{-1}) and IFNγ (100 u ml^{-1}) for 6 h. The means ± s.e.mean (vertical lines) are shown for 12 determinations performed on 4 separate experimental days. Cells were treated with the inhibitors 30 min before the addition LPS/IFNγ.

Cell viability, assessed by mitochondrial respiration, was not altered by the inhibitors at concentrations up to 3 mM (not shown).

Effects of mercaptoalkylguanidines on COX-2 activity in J774.2 cells: exogenous arachidonic acid stimulation

After stimulation of J774 cells with LPS (10 μg ml^{-1}) and IFNγ (100 u ml^{-1}), followed by wash-out and a 30 min incubation with arachidonic acid (15 μM), there was a marked increase in 6-keto-$PGF_{1\alpha}$ production (from 1.74 ± 0.44 to 20.44 ± 1.67 ng ml^{-1}, $P<0.01$). A significant, dose-dependent inhibition of prostaglandin production was induced by MEG, but not by the other NOS inhibitors tested (Figure 6).

Effects of mercaptoalkylguanidines on iNOS activity in J774.2 cells

In order to determine whether the inhibitory effect of MEG and similar agents on prostaglandin production is a consequence of the modification of NO synthesis (as previously observed; see Discussion), we also evaluated the effect of the various NOS inhibitors tested on nitrite production. LPS stimulation for 6 h induced a moderate accumulation of nitrite (8.3 ± 1.5 μM) that was inhibited by pretreatment with all the NOS inhibitors in a dose-dependent fashion (see Table 1 for IC_{50} values). However, while inhibition of NO synthesis by mercaptoalkylguanidines was accompanied by inhibition of prostaglandin production, reduction of nitrite levels by aminoguanidine, L-NMA and L-NAME was not paralleled by a reduction of prostaglandin levels.

Effects of indomethacin and NS-398 on COX-1 and COX-2 activity in HUVECs or J774.2 macrophages

In comparative experiments, arachidonic acid-stimulated HUVECs (exogenous arachidonic acid, 6 h stimulation) or immunostimulated J774.2 cells (endogenous arachidonic acid, 6 h incubation) were treated with indomethacin, a non selective COX-1 and COX-2 inhibitor, and NS-398, a selective COX-2 inhibitor (see: Discussion). In arachidonic acid-stimulated HUVECs, inhibition of COX activity with indomethacin inhibited prostaglandin formation with an IC_{50} value of 0.18 μM ($n=6$), whereas no significant inhibition by

Table 1 IC_{50} values for the inhibition of 6-keto-$PGF_{1\alpha}$ and nitrite production of stimulated J774.2 macrophages by mercaptoalkylguanidines, aminoguanidine, L-arginine derivatives, indomethacin and NS-398

Compound	*IC_{50} (μM)*	
	6-keto-$PGF_{1\alpha}$ production	*Nitrite production*
Mercaptoethylguanidine	33	16
N-methyl-mercaptoethyl-guanidine	34	61
N,N′-dimethyl-mercapto-propylguanidine	55	6
S-methyl-mercaptoethyl-guanidine	44	21
Guanidinoethyldisulphide	52	4
Aminoguanidine	ND	57
N^G-methyl-L-arginine	ND	71
N^G-nitro-L-arginine methyl ester	ND	226
Indomethacin	0.013	ND
NS-398	<0.001	ND

ND = there was no inhibition at 3 mM or the inhibition at 3 mM did not reach 50% and so an IC_{50} value could not be determined. Drugs were added to J774.2 cells 30 min before LPS and IFNγ.

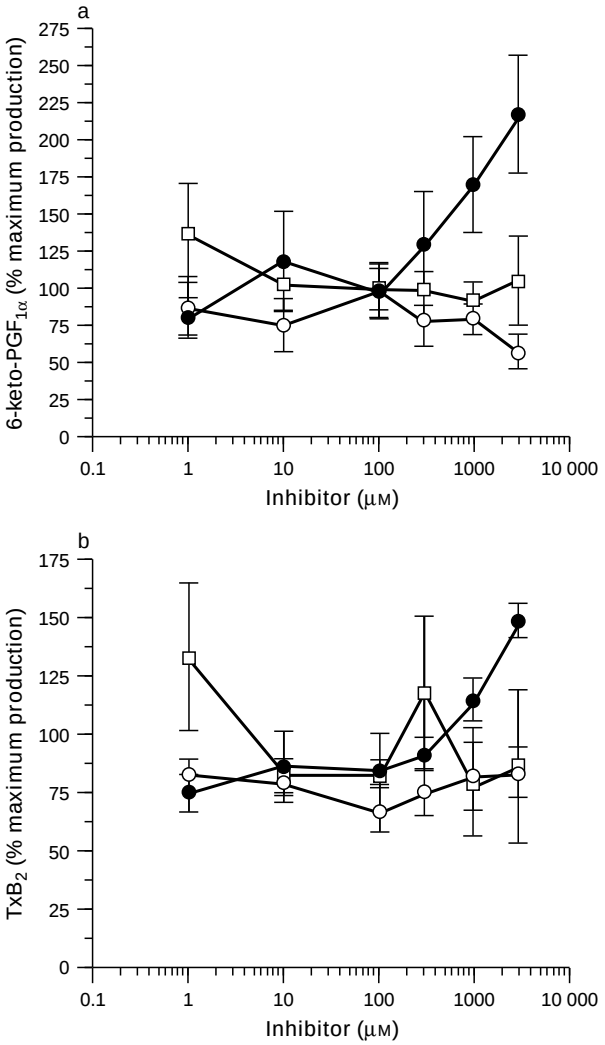

Figure 5 Effect of aminoguanidine (□), L-NAME (○) and L-NMA (●) (each at 1 μM – 3 mM) on 6-keto-$PGF_{1\alpha}$ (a) or TxB_2 (b) production in J774.2 macrophages exposed to LPS (10 μg ml^{-1}) and IFNγ (100 u ml^{-1}) for 6 h. The means ± s.e.mean (vertical lines) are shown for 12 determinations performed on 4 separate experimental days. Cells were incubated for 30 min with the inhibitors before the addition of LPS/IFNγ.

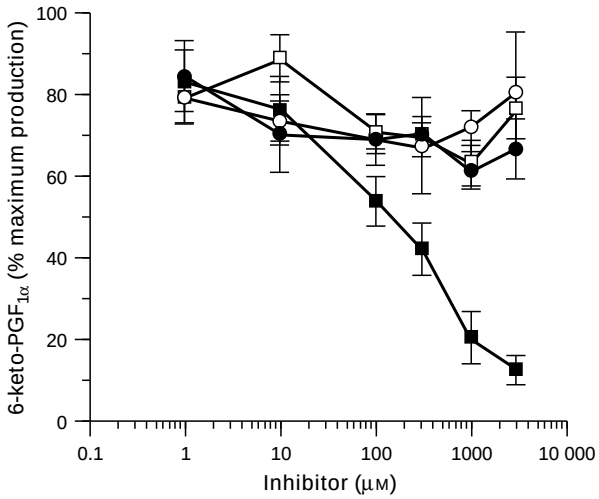

Figure 6 Effect of mercaptoethylguanidine (■), aminoguanidine (□), L-NAME (○) and L-NMA (●) (each at 1 μM – 3 mM) on 6-keto-$PGF_{1\alpha}$ production in arachidonic acid-stimulated J774.2 macrophages, which had previously been exposed to LPS (10 μg ml^{-1}) and IFNγ (100 u ml^{-1}) for 6 h. The means ± s.e.mean (vertical lines) are shown for 6 determinations performed on 2 separate experimental days. Cells were incubated for 30 min with arachidonic acid in the absence or presence of the inhibitors.

NS-398 was observed, up to 10 μM (not shown). In contrast, in immunostimulated J774 cells, the IC_{50} values for inhibition by indomethacin and NS-398 were 0.013 μM and <1 nM, respectively (Figure 7). Thus, our data show a more than 10,000 fold selectivity of NS-398 towards COX-2, and an approximately 10 fold selectivity of indomethacin towards COX-2 activity.

NS-398 and indomethacin did not affect NO production in the immunostimulated macrophages (Figure 7).

Effects of mercaptoethylguanidine, aminoguanidine, L-NMA and L-NAME on the activities of purified COX-1 and COX-2 enzymes

To confirm that the inhibition of prostaglandin metabolite production by mercaptoalkylguanidines observed in J774.2 cells was due to the direct inhibition by these compounds of COX enzymatic activity, experiments were performed with purified COX-1 and COX-2 enzymes. Similar to the its effect in J774.2 macrophages, MEG inhibited both COX-1 ($IC_{50} = 33$ μM) and COX-2 ($IC_{50} = 36$ μM) activities in a dose-dependent manner (Figure 8). At the highest concentrations (1 and 3 mM) aminoguanidine exhibited a moderate but significant inhibition of COX-1 ($P < 0.001$), but it had no effect on COX-2 activity. L-NAME did not affect the COX activity up to 1 mM. However, at 3 mM it caused approximately 50% inhibition of both enzymes. L-NMA (1 μM to 3 mM) did not alter PGH_2 production, either by COX-1 or by COX-2.

Discussion

In the present study, we have confirmed that endothelial cells in culture constitutively express COX-1, whereas J774.2 macrophages express COX-2 in response to immunostimulation (Mitchell *et al*., 1994). Moreover, our study demonstrated that the small but significant COX activity present in non-stimulated J774 macrophages is due to expression of COX-1 in this cell type. In a previous study, Mitchell *et al*. (1994) found a small amount of COX activity in unstimulated J774 cells and this was not characterized due to the cross-reactivity of the COX antibodies used in this study. Our data show not only

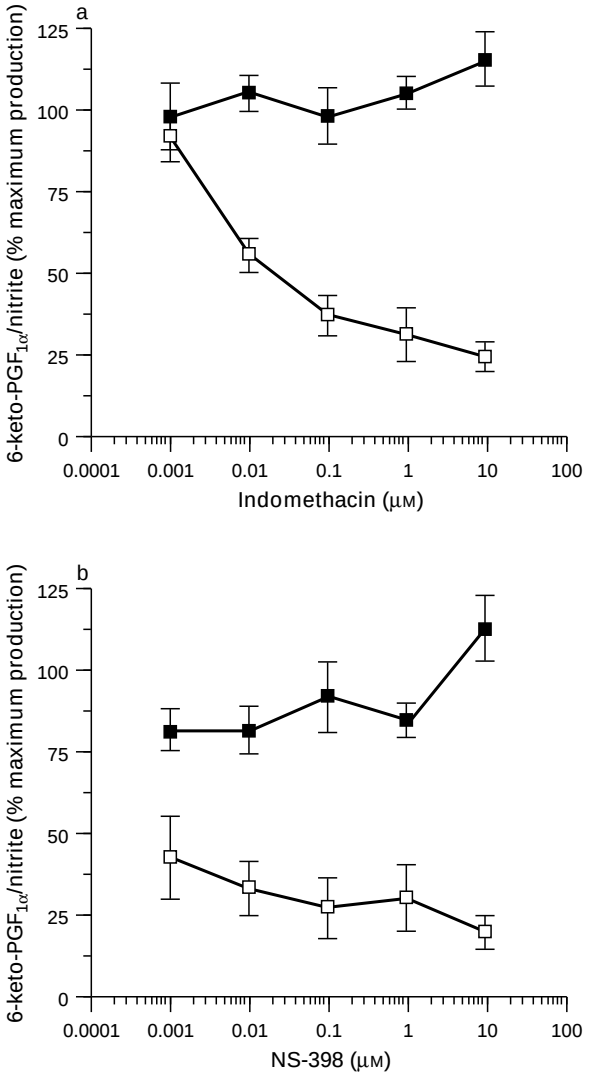

Figure 7 Effect of indomethacin (a) or NS-398 (b) on 6-keto-$PGF_{1\alpha}$ (□) and nitrite (■) production in J774.2 macrophages exposed to LPS ($10\,\mu g\,ml^{-1}$) and IFNγ ($100\,u\,ml^{-1}$) for 6 h. The means ± s.e.-mean (vertical lines) are shown for 9 determinations performed on 3 separate experimental days. Cells were incubated for 30 min with the inhibitors before the addition of LPS/IFNγ.

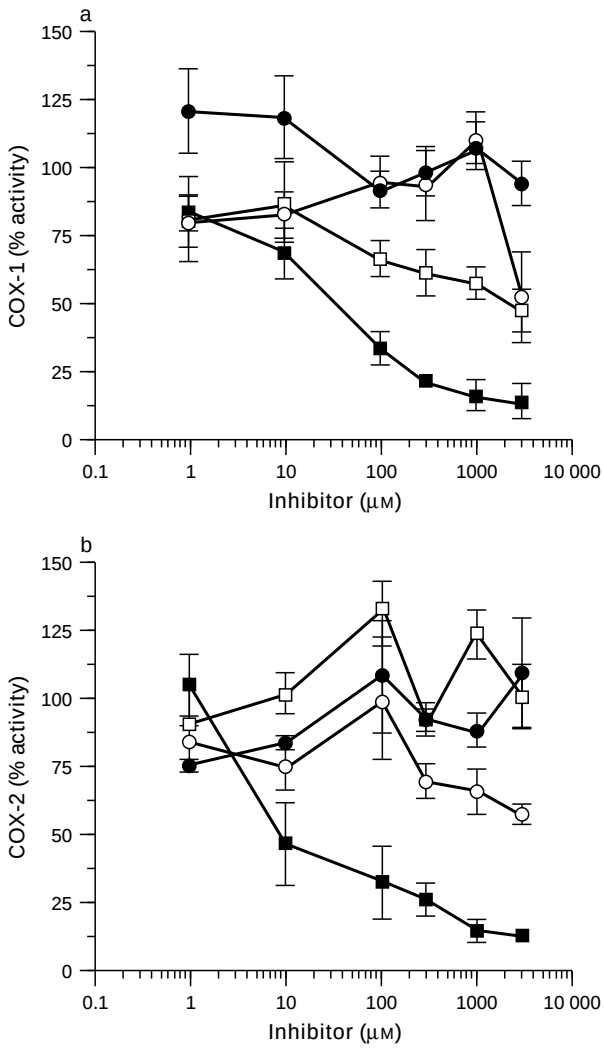

Figure 8 Effect of mercaptoethylguanidine (■), aminoguanidine (□), L-NAME (○) and L-NMA (●) (each at $1\,\mu M - 3\,mM$) on purified cyclo-oxygenase-1 (COX-1) (a) and COX-2 (b) activities. The means ± s.e.mean (vertical lines) are shown for 9 determinations performed on 3 separate experimental days. Enzyme activity was evaluated by measuring the release of $PGF_{2\alpha}$, the stable metabolite of PGH_2, in the reaction samples by Enzyme immunoassay.

that the COX isoform expressed by unstimulated J774 cells is COX-1, and not COX-2, but also suggest that immunostimulation of these cells results in a down-regulation of COX-1, with concomitant upregulation of COX-2. Similar 'isoform switch' has previously been obtained for the constitutive and inducible NOS isoforms in various cell types (Szabó, 1995).

In agreement with the results of the COX Western blots, we found arachidonic acid stimulated prostaglandin production in HUVECs and in J774 cells. Moreover, we found a significant increase in prostaglandin production in response to immunostimulation, either when endogenous arachidonic acid was used by the cells for prostaglandin production or, especially, when the cells were treated with exogenous arachidonic acid after immunostimulation. The finding that immunostimulation for 6 h caused an approximately 4 fold increase in prostaglandin production in the J774 cells is similar to the results of previous studies (Mitchell *et al*., 1993; Swierkosz *et al*., 1995), although in the present study, we found that the basal production of 6-keto-$PGF_{1\alpha}$ and TxB_2 was higher than that previously obtained (<0.3 ng ml^{-1}) for the same cell type (Mitchell *et al*., 1993; Swierkosz *et al*., 1995); this may be due to methodological differences. In addition, the increase in prostaglandin formation from endogenous arachidonic acid after 6 h of immunostimulation was lower than the increase seen at 12 or 24 h (Swierkosz *et al*., 1995). However, we chose a 6 h incubation period for immunostimulation, in order to avoid the interference of the production of large amounts of NO with COX (see: Introduction). We previously found that the increase in nitrite/nitrate production in LPS-stimulated J774 macrophages was marginal over the 6 h time period (Zingarelli *et al*., 1996).

Characterization of the various COX isoforms in the cell types used in the present study permitted the rational design of experimental protocols investigating the effect of mercaptoalkylguanidines and related compounds on the activities of COX-1 and COX-2 in intact cells. The effect of mercaptoalkylguanidines on COX-1 activity was studied in HUVECs stimulated with arachidonic acid for 6 h, as well as in J774 cells stimulated with arachidonic acid for 30 min. Exogenous arachidonic acid was necessary for the measurement of the effect of these agents on COX-1 activity, since in the absence of exogenous arachidonic acid, the amounts of arachidonic acid detected in the supernatant were not sufficient to study the

effects of the various inhibitors. Moreover, short incubation periods with arachidonic acid (30 min, for example), did not result in significant increases in the prostaglandin concentrations in the supernatant (unpublished data). Even 6 h of stimulation of the HUVECs with arachidonic acid only resulted in an approximately 2 fold increase in the prostaglandin concentrations in the supernatant, consistent with a lower prostaglandin output by cells expressing COX-1 when compared to cells expressing COX-2.

When designing experiments on the effects of mercaptoalkylguanidines on COX-2 activity, we used immunostimulated J774 macrophages, which show a marked expression of COX-2 over 6 h, as confirmed by Western blotting. We investigated the effect of mercaptoalkylguanidines on COX-2 activity both in response to endogenous and exogenous arachidonic acid stimulation. It is noteworthy that the immunostimulated J774 cells still expressed lower levels of COX-1. However, prostaglandin production by COX-1 may only contribute, to a small degree, to the net prostaglandin production in immunostimulated J774 cells for the following reasons: (i) in the absence of exogenous arachidonic acid, we found that prostaglandin production by COX-1 was minimal, and (ii) in immunostimulated cells, the amount of arachidonic acid stimulated prostaglandin production was 5 times higher than in resting J774 macrophages.

Our results demonstrate that mercaptoalkylguanidines, novel potent inhibitors of iNOS (Southan *et al*., 1996), also inhibit prostaglandin production *in vitro*. This effect was apparent, regardless of the method by which prostaglandin production was elicited: in response to exogenous arachidonic acid in endothelial cells or macrophages, in response to endogenous arachidonic acid in immunostimulated macrophages, or in response to exogenous arachidonic acid in immunostimulated macrophages. The IC_{50} for MEG varied from approx. 20 μM (arachidonic acid stimulated HUVECs) to approx. 200 μM (arachidonic acid stimulated immunostimulated J774 cells). These data suggest that MEG may have some preference towards the constitutive isoform of COX, although this preference may be related to differential cellular uptake of the compound, since the IC_{50} values on the purified enzymes were similar, and the IC_{50} values on arachidonic acid stimulated J774 cells (COX-1) and arachidonic acid stimulated J774 cells previously treated with LPS and IFN (COX-2) were comparable (60 μM and 200 μM, respectively). Nevertheless, the lack of marked isoform selectivity of MEG on COX is in contrast to the selective inhibitory effect of MEG on the inducible, but not the constitutive isoform of NOS (Southan *et al*., 1996). Moreover, we find that all mercaptoalkylguanidine derivatives tested (MEG, N-methyl-MEG, N,N′-dimethyl-mercaptopropyl-isothiourea, and guanidinoethyldisulphide) have similar potencies in inhibiting prostaglandin production (see Table 1 for IC_{50}). This is in contrast to the present (Table 1) and previous (Southan *et al*., 1996) data showing marked differences in the potencies of these compounds in inhibiting the activity of iNOS.

Although it has been proposed that in *in vitro* and *in vivo* models of inflammation inhibition of NOS decreases the production of prostaglandins by inhibiting NO synthesis (Sautebin & Di Rosa, 1994; Sautebin *et al*., 1995; Salvemini *et al*., 1993; 1994; 1995), the inhibitory effect on prostaglandin production of mercaptoalkylguanidines appears to be a direct effect on COX activity rather than a consequence of their ability to reduce NO synthesis. This hypothesis is supported by several findings: (i) inhibition of NOS activity by aminoguanidine, L-NMA and L-NAME was not paralleled by reduced prostaglandin levels; (ii) aminoalkylisothioureas and mercaptoalkylguanidines suppressed the release of 6-keto-$PGF_{1\alpha}$ and TxB_2 under experimental conditions in which arachidonic acid-stimulated HUVECs or J774 cells did not produce detectable levels of nitrite; (iii) MEG was a potent inhibitor of the enzymatic activity of purified COX-1 and COX-2 enzymes, ruling out the involvement of other cellular factors in the inhibition seen in the HUVECs and J774 cells.

On the one hand, at 1 – 3 mM, NOS inhibitors other than mercaptoalkylguanidines also had a modest direct inhibitory effect on COX activity, most notably, L-NAME and aminoguanidine on COX-1, and L-NAME on COX-2 (Figure 8). On the other hand, no marked inhibition of prostaglandin formation was observed with L-NAME or aminoguanidine in the intact cell studies, although, at the highest concentrations used, these agents did cause a substantial inhibition of nitrite production in the J774 cells. Taken together, these findings suggest the following: (1) at high concentrations, NOS inhibitors other than mercaptoalkylguanidines may also directly reduce the catalytic activity of COX enzymes; (2) when intact cells are treated with these NOS inhibitors, the intracellular concentrations of these inhibitors are not likely to reach high enough levels to exhibit a similar inhibitory effect; and (3) in the present system, NO plays a minor, if any, role in inducing prostaglandin formation by enhancing the catalytic activity of COX.

The demonstration that MEG and other mercaptoalkylguanidines suppress COX-2 as well as iNOS provides a potential explanation for the antiinflammatory properties of such inhibitors in models of shock. Administration of mercaptoalkylguanidines or their corresponding aminoalkylisothiourea precursors improved survival and cardiovascular performance, organ function and survival in endotoxic shock (Szabó *et al*., 1995; 1996; Thiemermann *et al*., 1995; Southan *et al*., 1996). Similarly, in a rodent model of haemorrhagic shock we have recently demonstrated that the beneficial pressor effect of MEG and the improvement of vascular reactivity is associated with a reduction of plasma levels of both prostaglandins and NO (unpublished observations).

The data presented here do not address the molecular mechanism by which mercaptoalkylguanidines inhibit COX function. The demonstration that isothioureas perturb the haeme spectra of NOS, suggests that they bind close to, or interact with, the haeme centre of NOS (Garvey *et al*., 1994). Since cyclo-oxygenases also possess a haeme at the active site which is essential for catalytic activity (Smith *et al*., 1991), a similar mechanism may apply. The similar inhibitory potencies of the various mercaptoalkylguanidines tested towards COX-1 and COX-2 activities, and the similar potencies of various substituted mercaptoalkylguanidines on COX-2 activity suggest that mercaptoalkylguanidines possess less stringent binding requirements for the inhibition of COX activity than for inhibition of NOS activity.

The proposal that mercaptoalkylguanidines inhibit COX by interfering with the iron-haem centre may also explain the lack of an inhibitory effect on COX activity of L-NAME, L-NMA (at concentrations up to 1 mM) and L-arginine analogues, which inhibit NOS activity mainly by occupation of the substrate binding site (for review see Southan & Szabó, 1996). However, non-specific properties of L-arginine analogues may include their inhibition of the activity of iron-containing enzymes (Peterson *et al*., 1992). In our study L-NMA, at the highest concentrations (1 and 3 mM) caused a significant increase in 6-keto-$PGF_{1\alpha}$ and TxB_2 release in the medium of LPS-stimulated J774.2 macrophages. This is in agreement with previous findings demonstrating that L-NMA, at inhibitory concentrations for nitrite production, caused a significant increase in the expression of COX-2 protein in J774 cells (Swierkosz *et al*., 1995). Conversely, in our experiments L-NAME 3 mM partially inhibited the activity of purified COX-1 and COX-2, but did not affect COX activity in HUVECs and J774 cells. It is possible that a near-complete reduction of NO production is required to see the inhibitory effect of NO on COX activity. It is also possible that there is a delicate balance between NO levels and non-specific actions of L-arginine based NOS inhibitors, when used at high concentrations.

Pathophysiological levels of NO and prostaglandins importantly contribute to the tissue damage of many inflammatory diseases, such as arthritis, ulcerative colitis, various cardiovascular diseases and circulatory shock. In these conditions, non-steroidal anti-inflammatory drugs, such as indomethacin and, more recently, NS-398, a selective inhibitor of COX-2 (Futaki *et al*., 1994), have been shown to exert anti-inflammatory effects by reducing prostaglandin release (Masferrer *et al*., 1994; Salvemini *et al*., 1995). However, as confirmed in our study, indomethacin and NS-398 are not able to inhibit NOS activity (Salvemini *et al*., 1995), and consequently the cytotoxicity mediated by NO. Therefore, a dual inhibitory mechanism of mercaptoalkylguanidines on COX-2 and iNOS may have therapeutic potential in pathophysiological conditions where both enzymes are co-expressed.

In conclusion, we have demonstrated that MEG and related mercaptoalkylguanidines are direct inhibitors of COX activity. By analogy with NOS, it seems reasonable to propose that their mechanism of action is related to interference with the iron-haeme centre of COX, although this possibility needs to be investigated directly. Taken together with results from previous studies showing selective inhibitory effects of mercaptoalkylguanidines on iNOS (Southan *et al*., 1996; Szabó *et al*., 1996), our data suggest that this class of agents, with dual inhibitory effects on NOS and COX, may have therapeutic potential in inflammatory disorders.

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