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Co-induction of nitric oxide synthase and cyclo-oxygenase: interactions between nitric oxide and prostanoids

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1 Lipopolysaccharide (LPS) co-induces nitric oxide synthase (iNOS) and cyclo-oxygenase (COX-2) in J774.2 macrophages. Here we have used LPS-activated J774.2 macrophages to investigate the effects of exogenous or endogenous nitric oxide (NO) on COX-2 in both intact and broken cell preparations. NOS activity was assessed by measuring the accumulation of nitrite using the Griess reaction. COX-2 activity was assessed by measuring the formation of 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) by radioimmunoassay. Western blot analysis was used to determine the expression of COX-2 protein. We have also investigated whether endogenous NO regulates the activity and/or expression of COX *in vivo* by measuring NOS and COX activity in the lung and kidney, as well as release of prostanoids from the perfused lung of normal and LPS-treated rats.

2 Incubation of cultured murine macrophages (J774.2 cells) with LPS (1 $\mu g\,ml^{-1}$) for 24 h caused a time-dependent accumulation of nitrite and 6-keto-$PGF_{1\alpha}$ in the cell culture medium which was first significant after 6 h. The formation of both 6-keto-$PGF_{1\alpha}$ and nitrite elicited by LPS was inhibited by cycloheximide (1 μM) or dexamethasone (1 μM). Western blot analysis showed that J774.2 macrophages contained COX-2 protein after LPS administration, whereas untreated cells contained no COX-2.

3 The accumulation of 6-keto-$PGF_{1\alpha}$ in the medium of LPS-activated J774.2 macrophages was concentration-dependently inhibited by chronic (24 h) exposure to sodium nitroprusside (SNP; 1–1000 μM). Sodium nitroprusside (1–1000 μM) also acutely (30 min) inhibited COX-2 activity in broken cell preparations of LPS-activated (12 h) J774.2 macrophages, in a similar concentration-dependent manner. Addition of adrenaline (5 mM) and glutathione (0.1 mM) increased the activity of COX-2 in broken cell preparations. In the presence of these co-factors, SNP inhibited prostanoid production only at the highest concentration used (1 mM). When J774.2 cells were incubated in the presence of LPS (1 $\mu g\,ml^{-1}$) and N^G-monomethyl-L-arginine (L-NMMA: 1 mM) for 12 h, SNP at the highest concentration used (1 mM) acutely (30 min) inhibited the activity of COX-2 in cell homogenates with co-factors. However, when J774.2 macrophages were incubated for 24 or 12 h with LPS (1 $\mu g\,ml^{-1}$) and L-NMMA (1 mM), the addition of SNP (0.001–1000 μM) increased in a concentration-dependent manner the accumulation of 6-keto-$PGF_{1\alpha}$ in intact cells (measured at 24 h) and COX-2 activity in cell homogenates in the presence of co-factors (determined at 12 h). SNP (1 mM; together with LPS for 12 h) decreased the amount of COX-2 protein induced by LPS in J774.2 macrophages.

4 Indomethacin (30 μM) abolished the formation of 6-keto-$PGF_{1\alpha}$ by LPS-activated macrophages, but had no effect on the release of nitrite. Conversely, L-NMMA, at the highest concentrations used (1 and 10 mM), increased the release of 6-keto-$PGF_{1\alpha}$, an effect which was reversed by excess L-arginine (3 mM) but not by D-arginine. Similarly, the decrease in nitrite formation caused by L-NMMA was partially reversed by L-arginine (3 mM), but not by D-arginine. L-NMMA (10 mM; together with LPS for 12 h) increased the amount of COX-2 protein induced by LPS in J774.2 macrophages.

5 In separate experiments, J774.2 macrophages were activated with LPS (1 $\mu g\,ml^{-1}$), and L-NMMA (10 mM) was added for various times (0.5–24 h) before the collection of medium at 24 h. L-NMMA enhanced the release of 6-keto-$PGF_{1\alpha}$ in a time-dependent manner, with the maximal enhancement seen when the NOS inhibitor was incubated with the cells for 24 h.

6 In experiments on male Wistar rats, we investigated the effect of L-NMMA on the release of prostanoids (6-keto-$PGF_{1\alpha}$, prostaglandin E_2, thromboxane B_2) elicited by arachidonic acid (AA, 30 nmol) from *ex vivo* perfused kidneys and lungs. The release from the organs from normal and LPS-treated rats was unaffected by L-NMMA intraperitoneally (30 $mg\,kg^{-1}$) for 6 h together with LPS (5 $mg\,kg^{-1}$) or LPS vehicle. Similarly, acute (15 min) *in vitro* exposure to L-NMMA (1 mM) of the perfused organs from control and LPS-treated animals did not change the release of prostanoids elicited by AA (30 nmol).

7 These results show that LPS causes the induction of iNOS and COX-2 in J774.2 macrophages. The co-release of NO and PGI_2 induced by LPS is dependent on protein synthesis and occurs after a lag-time of 6–12 h. The formation of COX metabolites has no effect on NOS activity whereas NO inhibits both COX-2 activity and induction. These results demonstrate that NOS and COX can be co-induced *in vitro* and that under these conditions large amounts of NO inhibit the degree of COX expression and activity. In the absence of endogenous NO, lesser amounts of exogenous NO increase the activity of COX-2. In those situations *in vivo* when the level of NO induction is relatively low, NO does not regulate the increased activity of COX.

Keywords: Nitric oxide; nitric oxide synthase; prostacyclin; cyclo-oxygenase; N^G-monomethyl-L-arginine; N^G-nitro-L-arginine; cytokine; endotoxin

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Introduction

Nitric oxide (NO) is formed from L-arginine by NO synthase (NOS) in a wide variety of cells (see Förstermann *et al.*, 1991; Nathan, 1992). Several isoforms of NOS have now been isolated, purified, cloned and expressed (see, Förstermann *et al.*, 1991; Janssens *et al.*, 1992; Lamas *et al.*, 1992; Lowenstein *et al.*, 1992; Nishida *et al.*, 1992). The constitutive isoform in endothelial cells (eNOS) is a 135 kD protein located predominantly in the membrane fraction (Pollock *et al.*, 1991; Sessa *et al.*, 1992). Bacterial lipopolysaccharide (LPS) or cytokines induce macrophages, vascular smooth muscle and other cells to express a different isoform of NOS (see Förstermann *et al.*, 1991; Nathan, 1992). The formation of large amounts of NO by the inducible isoform of NOS (iNOS) accounts for the cytotoxicity of activated macrophages and plays an important role in the circulatory failure associated with septic (Thiemermann & Vane, 1990; Julou-Schaeffer *et al.*, 1990; Wright *et al.*, 1992; Szabó *et al.*, 1993) and haemorrhagic shock (Thiemermann *et al.*, 1993). Although eNOS and iNOS have the same co-factor requirements for NADPH and tetrahydrobiopterin, and contain/require FAD/FMN (see Förstermann *et al.*, 1991; Pollock *et al.*, 1993), they differ in that eNOS is calcium-dependent, where iNOS is not. Both are inhibited, although to different degrees, by N^G-monomethyl-L-arginine (L-NMMA) or other N^G-substituted analogues of L-arginine (see Thiemermann, 1994).

Prostaglandins, a family of mediators, have numerous cardiovascular and inflammatory effects (see Botting & Vane, 1990). Cyclo-oxygenase (COX), the first enzyme in the pathway of prostaglandin and thromboxane A_2 formation from arachidonic acid, is localized primarily in the endoplasmic reticulum and exists as a dimer of 70 kD subunits (Helmer *et al.*, 1976). COX also exists in both constitutive (COX-1) and cytokine/LPS-inducible (COX-2) isoforms (see Xie *et al.*, 1992).

Endothelial cells contain eNOS (Pollock *et al.*, 1991; Sessa *et al.*, 1992) and COX-1 (Mitchell *et al.*, 1993a) and hence release both NO and prostacyclin (PGI_2) when activated by receptor-stimulating agonists such as bradykinin or ADP (De Nucci *et al.*, 1988; Mitchell *et al.*, 1991a; 1992). Similarly, induced iNOS (Stuehr *et al.*, 1991) and COX-2 may also co-exist in some cells after exposure to LPS or cytokines (Szabó *et al.*, 1993; Mitchell *et al.*, 1993a; Corbett *et al.*, 1993). In endothelial cells inhibition of NO release potentiates the coupled release of PGI_2 (Keen *et al.*, 1990), whereas exogenous NO (Doni *et al.*, 1988) or sodium nitroprusside (Keen *et al.*, 1990; Matthews *et al.*, 1993) inhibits the release of PGI_2. However, in other cell types, inhibitors of NO formation reduce the release of prostanoids (Stadler *et al.*, 1991; Rettori *et al.*, 1993; Corbett *et al.*, 1993; Salvemini *et al.*, 1993; 1994; Franchi *et al.*, 1994). We have, therefore, investigated how inhibition of endogenous NO formation in LPS-activated J774.2 macrophages affects COX-2 induction and the release of PGI_2. Furthermore, to investigate this crosstalk further, we have also examined whether inhibition of COX with indomethacin affects NO formation by these cells. Some of these results have been published in abstract form (Mitchell *et al.*, 1993b).

Methods

Cell Culture

Murine J774.2 macrophages were obtained from the European Collection of Animal Cell Culture (Salisbury, Wilts) and cultured in 96-well plates in Dulbecco's Modified Eagle's Medium (DMEM; ICN Biomedical Limited, High Wycombe, Bucks) containing 10% foetal calf serum (Gibco BRL, Uxbridge, Middlesex) and 2 mM L-glutamine (200 µl/well; B.D.H., Dagenham, Essex) until 80–100 % confluent. The culture medium was replaced with fresh medium at the beginning of each experiment. All drugs were dissolved in distilled water and sterilised by filtration through a 0.2 µm filter before being added to the cells under sterile conditions. Cells were incubated at 37°C in a humidified incubator.

Measurement of nitrite and 6-keto-$PGF_{1\alpha}$

Nitrite accumulation, an indicator of NO synthase activity, was measured in the culture medium by the Griess reaction adapted for a 96-well plate reader (Gross *et al.*, 1991). Nitrite was measured by adding 100 µl of Griess reagent (1% sulphanilamide and 0.1% naphthylethylenediamine in 5% phosporic acid) to 100 µl samples of cell culture medium. The optical density at 550 nm (OD_{550}) was measured with a Molecular Devices micro plate reader (Richmond, CA, U.S.A.). Nitrite concentrations were calculatd by comparison with the OD_{550} of standard solutions of sodium nitrite prepared in culture medium. The detection limit for nitrite measurement in culture medium was 1 µM. The accumulation of 6 keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$, the breakdown product of PGI_2), was measured as an indicator of COX activity by radioimmunoassay. [^{3}H]-6-keto-$PGF_{1\alpha}$ was obtained from Amersham International (Buckinghamshire). Antibodies to 6-keto-$PGF_{1\alpha}$ were purchased from Sigma Chemical Company (Poole, Dorset).

NOS and COX activity were induced in J774.2 macrophages by incubation with *E. coli* LPS (1 ng ml^{-1}–10 µg ml^{-1}) for 24 h. For time-course experiments, LPS was added for 0, 3, 6, 12 or 24 h, after which time the medium was removed for nitrite and 6-keto-$PGF_{1\alpha}$ determinations. In some experiments L-NMMA (0.1 µM – 10 mM; Calbiochem, La Jolla, CA, U.S.A.), D-NMMA (0.1 µM – 10 mM), dexamethasone (1 µM), sodium nitroprusside (0.001–1000 µM), indomethacin (30 µM) or cycloheximide (1 µM) were added together with LPS for 24 h after which time the medium was removed for the measurement of nitrite or 6-keto-$PGF_{1\alpha}$.

Broken cell preparations

J774.2 macrophages were cultured in T175 flasks until confluent. LPS (1 µg ml^{-1}) was added for 12 h, after which time the cells were washed and scraped into ice-cold phosphate buffered saline (pH 7.4). The cells were then centrifuged (1,000 *g* for 10 min) and the cell pellet was homogenized with a glass-Teflon homogeniser in Tris buffer (50 mM; pH 7.4) containing phenylmethylsulphonyl fluoride (1 mM), pepstatin A (1.5 mM) and leupeptin (0.2 mM). The broken cell preparation was incubated at 37°C with sodium nitroprusside (0.1–1000 µM) for 30 min. Arachidonic acid (30 mM) was added and the incubations continued for a further 15 min until stopped by boiling. The incubates were centrifuged (10,000 *g* for 30 min) and the concentrations of 6-keto-$PGF_{1\alpha}$ measured in the supernatant as indicators of COX activity. In separate experiments, COX activity was measured in the presence of the co-factors adrenaline (5 mM) and glutathione (0.1 mM) together with different concentrations of sodium nitroprusside (0.1–1000 µM) as above. In some experiments, J774.2 cells were incubated for 12 h with LPS (1 µg ml^{-1}) and L-NMMA (1 mM), in the presence or absence of SNP (0.01–1000 µM), and the activity of COX in cell homogentates was measured as described above.

Western blot analysis

J774.2 macrophages were cultured in T175 flasks until confluent. Cells were treated with LPS (1 µg ml^{-1}) and either L-NMMA (10 mM) or SNP (1 mM) or vehicle (distilled water) for 12 h. Cells were washed with PBS (pH 7.4) and incubated (10 min) with 2–3 ml of extraction buffer [Tris, 50 mM; EDTA, 10 mM; Triton X-100, 1 % v/v; phenylmethylsul-

phonyl fluoride (PMSF; 1 mM), pepstatin A (50 μM) and leupeptin (0.2 mM)] with gentle shaking. The cell extract was then boiled (10 min) in a ratio of 1:1 with gel loading buffer (Tris, 50 mM; SDS, 10 %; glycerol, 10 % and bromphenol blue, 2 mg ml^{-1}). The samples were loaded onto gradient gels (4–12 % Tris-glycine; Novex) and subjected to electrophoresis (1.5 h at 125 mV). The separated proteins were transferred to nitrocellulose (BIORAD; 1 h at 200 mV). After transfer to nitrocellulose, the blot was primed with a selective rabbit antibody raised to murine COX-2 (Cayman Chemical Company, MI, U.S.A.). The blot was then incubated with an anti-rabbit IgG developed in sheep, which was linked to alkaline phosphatase conjugate. The blot was then developed with premixed solution (pH 9.2) containing 5-bromo-4-chloro-3-indolyl phosphate (0.56 mM), nitro blue tetrazolium (0.48 mM), Tris (10 mM) and $MgCl_2$ (59.3 mM).

Measurement of cell viability

Cell respiration, an indicator of cell viability, was assessed by the mitochondrial dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan (Gross *et al.*, 1991). Cells in 96-well plates were incubated at 37°C with MTT (200 μg ml^{-1}) for 60 min. Culture medium was removed by aspiration and the cells solubilized in dimethylsulphoxide (DMSO; 100 μl). The extent of reduction of MTT to formazan within cells was quantitated by measurement of OD_{550}.

Whole organ experiments

Interactions between the NOS and COX systems were investigated in male Wistar rats (250–300 g). Rats were injected intraperitoneally (i.p.) with LPS (5 mg kg^{-1}) or vehicle and 6 h later killed by an overdose of pentobarbitone, at which time kidneys or lungs were removed and perfused *ex vivo*. Kidneys were perfused through the renal artery at 10 ml min^{-1}, whereas lungs were inflated via the trachea and perfused through the pulmonary artery at 5 ml min^{-1}, with warmed and oxygenated Krebs solution. The Krebs solution had the following composition (mM): NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.17, $CaCl_2$ 2.5, $NaHCO_3$ 2.5 and glucose 5.6, pH 7.4. After an equilibration period of 30 min, organs were infused with L-NMMA (1 mM) or vehicle and 15 min later arachidonic acid (AA, 30 nmol) was injected. Effluent was collected at intervals of 1 min. The concentrations of 6-keto-$PGF_{1\alpha}$, prostaglandin E_2 and thromboxane B_2 in the effluent were determined by radioimmunoassay. In separate experiments, rats were injected i.p. with L-NMMA (30 mg kg^{-1}) at the same time as LPS (5 mg kg^{-1}) or vehicle, and then at 6 h organs taken and perfused *ex vivo* as above. The activity of NOS was determined *ex vivo* by the ability of organ homogenates to convert [^{3}H]-L-arginine to [^{3}H]-L-citrulline (Mitchell *et al.*, 1991b). iNOS was distinguished from the constitutive NOS by performing experiments in the presence of Ca^{2+} (2 mM) or in the absence of Ca^{2+} (EGTA, 1 mM). COX activity was determined by the ability of organ homogenates to metabolize AA (30 μM) to prostacyclin (measured as 6-keto-$PGF_{1\alpha}$) as measured by radioimmunoassay.

Statistics

Results are shown as mean ± s.e.mean for n experiments. One way analysis of variance was used and P-values of $P<0.05$ (* or +), $P<0.01$ (**) or $P<0.001$ (***) taken as significant.

Materials

All compounds were obtained at the highest quality from Sigma Chemical Company, Poole, Dorset, unless otherwise stated.

Results

Accumulation of nitrite

Bacterial LPS (1 ng ml^{-1} – 10 μg ml^{-1}; for 24 h) caused concentration-dependent increases in nitrite in the culture medium of J774.2 macrophages ($n=9$ for each, data not shown), with a maximum effect being caused by 1 μg ml^{-1} of LPS. As treatment of the cells with this concentration of LPS did not affect cell viability ($n=3$; data not shown) and also caused maximal stimulation of COX-2 formation, LPS was used at this concentration in all further experiments.

Activation of macrophages with LPS (1 μg ml^{-1} for 24 h) caused a time-dependent increase in nitrite, which was significant at 12 h and 24 h (Figure 1). Dexamethasone (1 μM) or cycloheximide (1 μM), added 30 min prior to LPS, attenuated the increase measured at 24 h by 89 ± 5 % and 92 ± 4 %, respectively ($n=3$ for each).

Accumulation of 6-keto-$PGF_{1\alpha}$

Treatment of J774.2 macrophages with LPS (1 μg ml^{-1}) caused a time-dependent accumulation of 6-keto-$PGF_{1\alpha}$ in the culture medium which was first significant ($P<0.05$) at 6 h after the addition of LPS (Figure 1; $n=9$). The formation of 6-keto-$PGF_{1\alpha}$ at 24 h was attenuated by dexamethasone (1 μM; 80 ± 3 %) or cycloheximide (1 μM; 94 ± 4 %), added 30 min before LPS ($n=3$). Indomethacin (30 μM) abolished the release of 6-keto-$PGF_{1\alpha}$ at all time points, but did not affect nitrite release from LPS-activated cells, measured after 3, 6, 12 or 24 h ($n=9$ for each; data not shown).

Effect of sodium nitroprusside on the release of 6-keto-$PGF_{1\alpha}$

Sodium nitroprusside (0.1–1000 μM), added together with LPS for 24 h, produced a concentration-dependent inhibition of the formation of 6-keto-$PGF_{1\alpha}$ by J774.2 cells (Figure 2a; $n=12$) with no significant effect on cell viability (data not shown). A broken cell preparation of COX-2 (LPS-activated J774 macrophages) was used to assess any direct effects of SNP on COX-2 activity. Broken cell preparations of J774.2 macrophages exposed to LPS (1 μg ml^{-1} for 12 h) converted arachidonic acid (30 μM) to 6-keto-$PGF_{1\alpha}$; an effect that was inhibited by more than 80 % by indomethacin (30 μM; data not shown). Incubations of broken cell preparations with sodium nitroprusside (0.1–1000 μM) for 30 min produced a

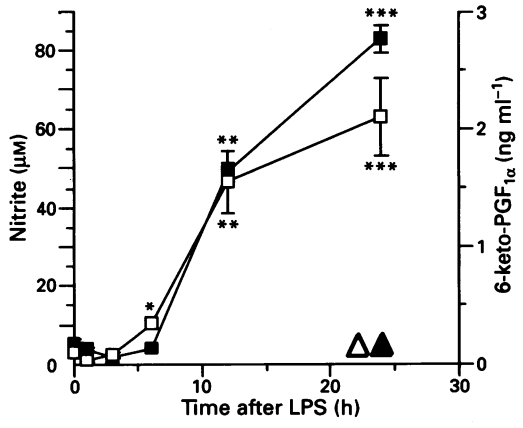

Figure 1 Time-course for the accumulation of nitrite (■) and 6-keto-$PGF_{1\alpha}$ (□) in the culture medium of J774.2 macrophages incubated with LPS (1 μg ml^{-1}). The amounts of nitrite (▲) or 6-keto-$PGF_{1\alpha}$ (Δ) in the culture medium of untreated cells after 24 h incubation are indicated. Each point represents the mean ± s.e.mean for 12 determinations performed on 4 separate experimental days. Significance of asterisks, see text.

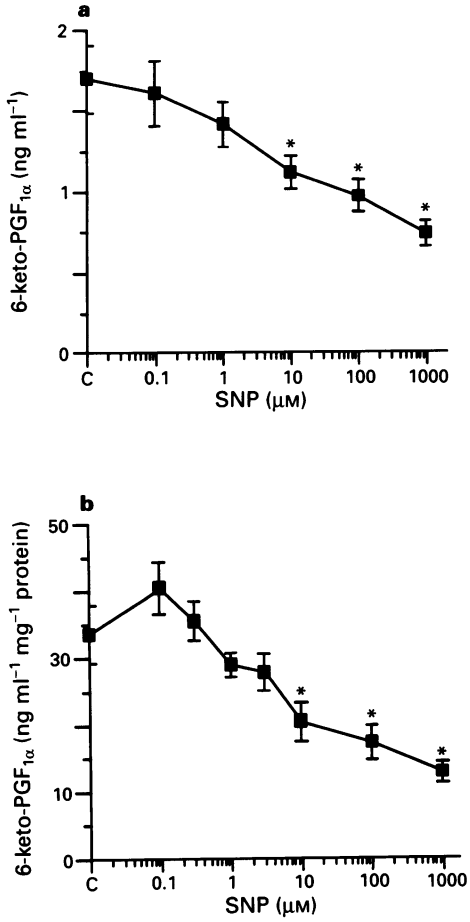

Figure 2 The effect of sodium nitroprusside on the accumulation of 6-keto-$PGF_{1\alpha}$ (a) or COX-2 activity (b) in J774.2 macrophages exposed to LPS (1 μg ml^{-1}) for 24 or 12 h, respectively. (a) Sodium nitroprusside (SNP; 0.1–1000 μM), co-incubated with LPS for 24 h, caused a concentration-dependent inhibition of 6-keto-$PGF_{1\alpha}$ accumulation in the medium of intact J774.2 macrophages. The figure shows the mean ± s.e.mean for 12 determinations performed on 4 separate experimental days. (b) SNP (0.1–1000 μM), incubated for 30 min with homogenates of LPS-treated (12 h) J774.2 macrophages, concentration-dependently inhibited the ability of cell homogenates to form 6-keto-$PGF_{1\alpha}$ in response to exogenous arachidonic acid (30 μM). The data represents the mean ± s.e.mean from 12 determinations performed on 4 separate enzyme preparations. *Denotes significant differences in the production of 6-keto-$PGF_{1\alpha}$ (compared to control [C]-cells treated in the absence of SNP). Similar results were obtained when PGE_2 was measured as an index of COX-2 activity ($n = 12$; data not shown).

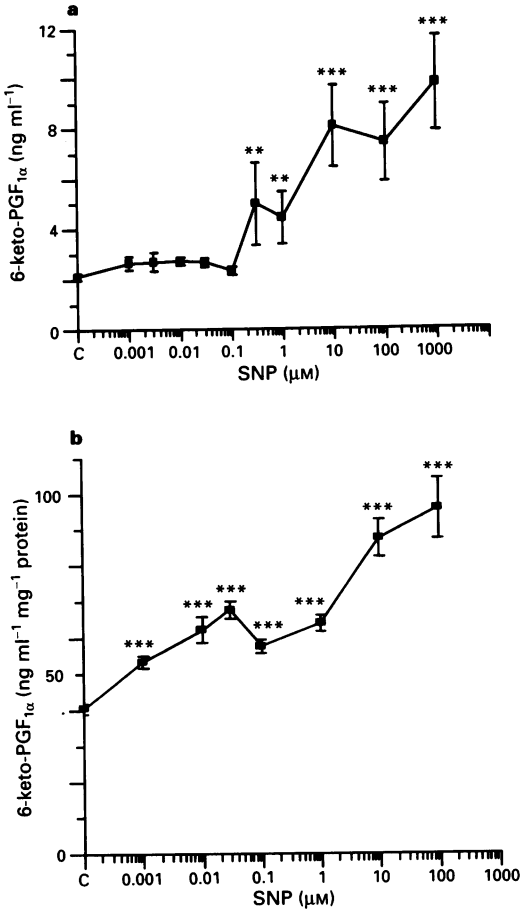

Figure 3 The effect of sodium nitroprusside on the accumulation of 6-keto-$PGF_{1\alpha}$ (a) or COX-2 activity (b) in J774.2 macrophages exposed to LPS (1 μg ml^{-1}) for 24 or 12 h, respectively, in the presence of L-NMMA (1 mM). (a) Sodium nitroprusside (SNP; 0.001–1000 μM), co-incubated with the cells in the presence of LPS (1 μg ml^{-1}) and L-NMMA (1 mM) for 24 h, caused a concentration-dependent increase in the accumulation of 6-keto-$PGF_{1\alpha}$ in the medium of intact J774.2 macrophages. The figure shows the mean ± s.e.mean ($n = 8$). (b) SNP (0.01–1000 μM), co-incubated with the cells in the presence of LPS (1 μg ml^{-1}) and L-NMMA (1 mM) for 12 h, caused a concentration-dependent increase in the ability of cell homogenates to form 6-keto-$PGF_{1\alpha}$ in response to exogenous arachidonic acid (30 μM) administered with the co-factors (adrenaline, 5 mM; glutathione, 0.1 mM). The figure shows the mean ± s.e.mean ($n = 9$). *Denotes significant differences in the production of 6-keto-$PGF_{1\alpha}$ (compared to control [C]-cells treated in the absence of SNP).

concentration-dependent inhibition of COX-2 activity which was significant at 10, 100 and 1000 μM (Figure 2b; $n = 12$). When haemoglobin (10 μM) was added together with SNP, only the highest concentration used caused a significant inhibition of COX-2 activity ($n = 6$; data not shown). COX-2 activity was greatly enhanced when the co-factors, adrenaline (5 mM) and glutathione (0.1 mM), were added to the incubations. This combination of co-factors maximally potentiates the activity of COX (Swierkosz, unpublished observations). SNP at the highest concentration of 1 mM also inhibited COX-2 activity in broken cell preparations carried out in the presence of adrenaline and glutathione ($n = 12$; data not shown).

When J774.2 macrophages were incubated with LPS (1 μg ml^{-1}) and L-NMMA (1 mM) for 12 h, SNP at the highest concentration used (1 mM; $n = 6$; data not shown) acutely (30 min) inhibited in the presence of co-factors the activity of COX-2 in cell homogenates. However, when J774.2 macrophages were incubated for 24 h with LPS (1 μg ml^{-1}) and L-NMMA (1 mM), the addition of SNP (0.001–1000 μM) to the culture medium for 24 h increased in a concentration-dependent manner the accumulation of 6-keto-$PGF_{1\alpha}$ in the medium of intact cells (Figure 3a; $n = 8$-32). Similarly, when J774.2 macrophages were incubated for 12 h with LPS (1 μg ml^{-1}) and L-NMMA (1 mM), the addition of SNP (0.01–1000 μM) to the culture medium for 12 h increased in a concentration-dependent manner the activity of COX-2 in cell homogenates containing co-factors (Figure 3b; $n = 9$). In the J774.2 cells which were not treated with LPS, production of 6-keto-$PGF_{1\alpha}$ was undetectable but became detectable when the cells were incubated with SNP (1 mM; $n = 4$; data not shown).

Effect of NO synthase inhibition on the accumulation of nitrite and 6-keto-$PGF_{1\alpha}$

L-NMMA (1 μM – 10 mM) caused a concentration-dependent inhibition of nitrite accumulation and a concentration-

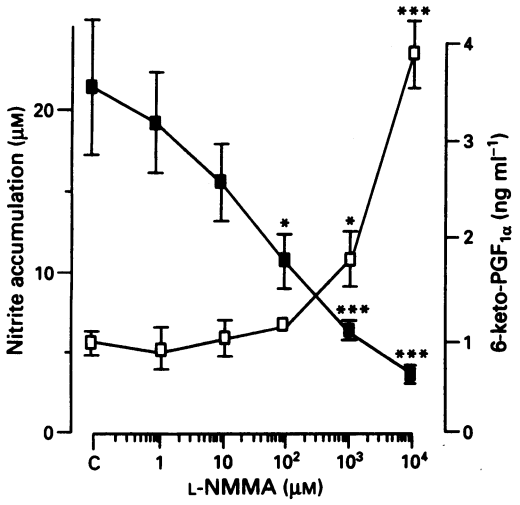

Figure 4 The effect of L-NMMA on nitrite (■) and 6-keto-$PGF_{1\alpha}$ (□) accumulation in the culture medium of J774.2 macrophages treated with LPS (1 μg ml^{-1}). L-NMMA was added together with LPS and incubated for 24 h. The points represent the mean ± s.e.mean for 9 determinations performed on 3 experimental days. *Denotes significant differences in the production of 6-keto-$PGF_{1\alpha}$ or in the production of nitrite (compared to control [C]-cells treated with LPS alone).

Table 1 Reversal by L-arginine of the effects of N^G-monomethyl-L-arginine (L-NMMA) on the formation of nitrite and 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) in LPS-treated (1 μM for 24 h) J774.2 macrophages

	LPS	*LPS + L-NMMA*	*LPS + L-NMMA + L-Arg*
Nitrite (μM)	49 ± 1	8 ± 1***	20 ± 3+
6-keto-$PGF_{1\alpha}$ (ng ml^{-1})	1.7 ± 0.1	3.1 ± 0.3*	1.7 ± 0.2+

L-Arginine (L-Arg 3 mM) reversed by approximately 50% the inhibitory effect of L-NMMA (1 mM) on the formation of nitrite and completely reversed the potentiation induced by L-NMMA (1 mM) on the formation of 6-keto-$PGF_{1\alpha}$. The data show the mean ± s.e.mean ($n = 3$). *Denotes significant differences between the LPS + L-NMMA group and the LPS group. +Denotes significant differences between the LPS + L-NMMA + L-Arg group and the LPS + L-NMMA group.

dependent increase in 6-keto-$PGF_{1\alpha}$ accumulation (Figure 4; $n = 9$). The inhibitory effect of L-NMMA (1 mM) on nitrite release from LPS-activated J774.2 macrophages was partially reversed by L-arginine (3 mM; Table 1; $n = 3$). Similarly, the potentiation of the release of 6-keto-$PGF_{1\alpha}$ from LPS-activated J774.2 macrophages caused by L-NMMA was prevented by L-arginine (3 mM; Table 1; $n = 3$). In contrast, D-NMMA (1 μM – 10 mM) had no significant effect on the LPS-stimulated formation of either nitrite or 6-keto-$PGF_{1\alpha}$ ($n = 3$; data not shown).

In separate experiments, J774.2 macrophages were activated with LPS (1 μg ml^{-1}; for 24 h), and L-NMMA (10 mM) was added to the cells for various times (0.5–24 h) before the collection of medium at 24 h (Figure 5). Addition of L-NMMA for 30 min before the collection of medium at 24 h after LPS did not affect the release of 6-keto-$PGF_{1\alpha}$ (Figure 5). However, addition of L-NMMA for 12, 18, 21 and 24 h caused significant and time-dependent increases in 6-keto-$PGF_{1\alpha}$ accumulation measured at 24 h. The maximal (3 fold) enhancement was seen when L-NMMA was incubated with LPS for the entire 24 h period (Figure 5; $n = 9$).

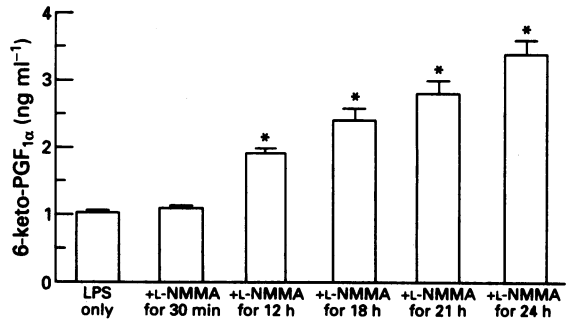

Figure 5 Accumulation of 6-keto-$PGF_{1\alpha}$ in LPS-treated J774.2 macrophages after incubation with L-NMMA for various times before the collection of medium. L-NMMA caused a time-dependent increase in 6-keto-$PGF_{1\alpha}$ accumulation when J774.2 macrophages were treated with LPS (1 μg ml^{-1}) for 24 h and L-NMMA (10 mM) (30 min–24 h) before the collection of medium. The enhancement by L-NMMA of the release of 6-keto-$PGF_{1\alpha}$ was maximal when L-NMMA was incubated with LPS for the entire 24 h period. The data represent the mean ± s.e.mean of 9 separate determinations. *Denotes significant differences from control (cells incubated with LPS only).

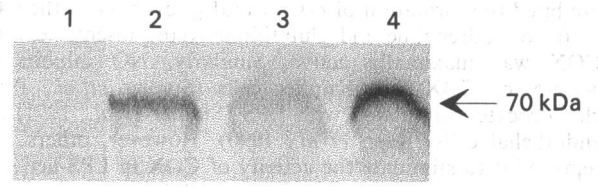

Figure 6 Effect of sodium nitroprusside (SNP) and L-NMMA on COX-2 induction in LPS-activated J774.2 macrophages. Cell extracts from untreated J774.2 macrophages contained no COX-2 (lane 1). However, extracts of J774.2 macrophages exposed to LPS (1 μg ml^{-1}) for 12 h contained COX-2 which migrated as a protein of approximately 70 kDa (lane 2). The amount of COX-2 present 12 h after LPS administration was decreased when SNP (1 mM) was co-incubated with LPS (lane 3) and increased when L-NMMA (10 mM) was co-incubated with the LPS (lane 4). The figure represents the data from a single experiment. Similar results were obtained with cell extracts in 5 other experiments.

Effect of SNP and L-NMMA on induction of COX-2 protein

Cell extracts from J774.2 macrophages incubated with LPS (1 μg ml^{-1} for 12 h) contained COX-2 protein ($n = 6$, Figure 6). The amount of COX-2 protein induced by LPS was increased approximately 2 fold by L-NMMA (10 mM, $n = 6$) and decreased by SNP (1 mM, $n = 3$; Figure 6). No COX-2 protein was detected in unstimulated cells ($n = 6$, Figure 6).

Effect of L-NMMA in whole organs

In the lung, LPS (5 mg kg^{-1}, i.p., for 6 h) increased NOS and COX activity, as measured in organ homogenates, as well as the release of thromboxane B_2 measured from perfused organs (NOS activity, pmol L-Cit $30\ min^{-1}\ mg^{-1}$ protein: control, 2.7 ± 0.6; LPS, 28 ± 20; COX activity, ng 6-keto-$PGF_{1\alpha}$ $30\ min^{-1}\ mg^{-1}$ protein: control, 14.7 ± 3.3; LPS, 30 ± 2.9; thromboxane release, ng ml^{-1} $5\ min^{-1}$: control, 5.93 ± 1.6; LPS, 10.1 ± 0.9). L-NMMA (30 mg kg^{-1}), given i.p. together with LPS (5 mg kg^{-1}) for 6 h or administered to perfused organs acutely for 15 min, did not affect any of the parameters measured above. Intraperitoneal (i.p.) administration of LPS (5 mg kg^{-1}, for 6 h) to rats increased the basal perfusion pressure of the kidney measured *ex vivo* (control, 36 ± 4; LPS, 63 ± 5 mmHg) but this was not associated with

any significant changes in the release of prostaglandins elicited by 30 nmol AA ($n = 3$).

Discussion

Here we show that endogenous or exogenous NO inhibits the expression of the COX-2 enzyme and that exogenous NO also inhibits its activity.

Sodium nitroprusside attenuated the accumulation of 6-keto-$PGF_{1\alpha}$ in the medium of intact J774.2 macrophages and reduced the expression of COX-2 protein within the same cells. This accords directly with the known inhibitory effects of SNP or NO on the release of PGI_2 from endothelial cells (Doni *et al.*, 1988; Keen *et al.*, 1990; Mathews *et al.*, 1993). More interestingly, endogenous NO had a similar inhibitory role in the cells, for the addition of L-NMMA, at a concentration that reduced the accumulation of nitrite from approximately 20 μM to 1 μM, caused a significant increase in PGI_2 release and an increase in the expression of COX-2 protein.

In addition to the inhibitory effect of NO on the expression of COX-2, we also most generally found it to reduce the activity of COX-2. For instance, in homogenates of cells treated with LPS for 12 h, addition of SNP for 30 min inhibited the formation of 6-keto-$PGF_{1\alpha}$, even when the COX co-factors adrenaline and glutathione were present, i.e. when COX was maximally active. Similarly, NO inhibits the activity of COX-2 in Kupffer cells (Stadler *et al.*, 1993), chondrocytes stimulated with LPS (Stadler *et al.*, 1991) and endothelial cells (Keen *et al.*, 1990). However, others have reported it to stimulate the activity of COX in LPS-activated RAW macrophages (Salvemini *et al.*, 1993), vascular smooth muscle cells (Inoue *et al.*, 1993), homogenates of COX-1 or COX-2 transfected cells (Salvemini *et al.*, 1993), islets of Langerhans (Corbett *et al.*, 1993), hypothalamic neurones (Rettori *et al.*, 1993), uterine smooth muscle (Franchi *et al.*, 1994) and chondrocytes stimulated with LPS and cytokines (Stadler *et al.*, 1991). None of these results distinguishes between the activity of COX and the expression of the enzyme. How can we explain these apparent conflicts? Perhaps there are different amounts of NO in the various assay systems used, as L-NMMA, for instance, suppresses the release of PGE_2 from interleukin-1-treated chondrocytes, when endogenous NO production is low, but elevates it when endogenous NO production is high (Stadler *et al.*, 1991). In our experiments, the endogenous production of NO by J774.2 macrophages certainly attained levels that inhibit the activity of COX-2. When the cells were co-incubated with LPS plus L-NMMA, to eliminate endogenously produced NO, together with SNP to provide controlled amounts of exogenous NO (Feelisch, 1991), we found that it enhanced the accumulation of 6-keto-$PGF_{1\alpha}$ measured at 24 h, and increased the acute formation of 6-keto-$PGF_{1\alpha}$ at 12 h after the addition of LPS.

The influence of NO on prostanoid synthesis may, therefore, vary in different cell types with variations in the rate of prostanoid synthesis, the state of cell activation, and the amount of iNOS and COX-2 proteins present. This hypothesis also answers the long standing controversy of whether nitrovasodilators release prostacyclin from the endothelium (Mehta *et al.*, 1983a,b; Schrör *et al.*, 1988; Levin *et al.*, 1981; Nakabayashi *et al.*, 1985) or not (Mehta *et al.*, 1983b; Fitzgerald *et al.*, 1984; Levin *et al.*, 1982; De Caterina *et al.*, 1985; Bennett *et al.*, 1987; Brotherton, 1986).

Thus, NO has a dual (potentiating in small amounts and inhibiting in large amounts) effect on the activity of COX-2 and an inhibitory effect on the expression of COX-2 protein. Mechanistically, this inhibition of COX-2 activity can be explained by the ability of NO to reduce the ferric-active form of COX to the ferrous-inactive form (Kanner *et al.*, 1992), or to nitrosylate tyrosine groups within COX (Shimokawa *et al.*, 1990). Conversely, there is not a good mechanistic explanation for the suggestion that NO directly stimulates the activity of COX (Stadler *et al.*, 1991; Corbett *et al.*, 1993; Rettori *et al.*, 1993; Salvemini *et al.*, 1993; Franchi *et al.*, 1994).

The dual modulatory effects of NO on COX activity may also be related to the levels of other radicals formed by macrophages, such as superoxide anions or hydrogen peroxide (Beckman *et al.*, 1990; Heinzel *et al.*, 1992). The concentrations of these agents will also vary greatly between different cell types, providing an alternative explanation of the conflicting observations described above (see Lands, 1985).

In contrast to the inhibitory effects of endogenously released NO on COX-2, the accompanying release of prostanoids did not affect the activity of NOS, even though exogenously applied PGE_2 or iloprost (a stable analogue of PGI_2) inhibit nitrite accumulation in LPS-treated J774.2 cells (Marotta *et al.*, 1992). This difference is explained by the endogenous release of prostanoids from the J774.2 macrophages being insufficient to produce this effect.

We also investigated the interactions between the NOS and COX systems *in vivo*, where, for instance, in septic or haemorrhagic shock elevated levels of cytokines lead to the induction of NOS and COX, and the formation of large amounts of NO (Thiemermann *et al.*, 1993). No evidence was found, however, for any cross regulation in our *ex vivo* organ studies, although this does not preclude the possibility that in other situations, when the induction of iNOS is more pronounced, NO may influence COX. For instance, in lungs from LPS-treated rats (Sautebin & Di Rosa, 1994) or in the rabbit perfused hydronephrotic kidney (Salvemini *et al.*, 1994), the activity of COX is stimulated by endogenous NO, although the mechanism of this effect is unclear. The lack of a regulatory effect of NO on the activity of COX found in our whole organ study is in line with the recent findings of Tsai *et al.* (1994) who questioned the existence of significant interactions of the COX haem with NO under conditions likely to be encountered *in vivo*. They showed, using electron paramagnetic resonance measurements, that NO reacted strongly with COX-1, forming an NO-haem complex and so breaking the bond between the protein and the haem iron. However, this was only in anaerobic conditions, which would not allow normal cyclo-oxygenase or peroxidase catalysis.

We conclude that iNOS and COX-2 can be co-induced *in vitro* and that under these conditions NO inhibits the degree of both COX-2 expression and activity. In the absence of endogenous NO, exogenous NO can activate COX-2. In situations *in vivo*, in which the level of NO induction is relatively low, NO does not regulate the increased activity of COX.

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