Regulation of prostaglandin production by nitric oxide; an *in vivo* analysis

¹Daniela Salvemini, Steven. L. Settle, Jaime. L. Masferrer, Karen Seibert, Mark. G. Currie & Philip Needleman

G.D. Searle, Department of Molecular Pharmacology, 800 N. Lindbergh Boulevard, St. Louis, MO 63167, U.S.A.

1 Endotoxin *E. Coli* lipopolysaccharide (LPS)-treatment in conscious, restrained rats increased plasma and urinary prostaglandin (PG) and nitric oxide (NO) production. Inducible cyclo-oxygenase (COX-2) and nitric oxide synthase (iNOS) expression accounted for the LPS-induced PG and NO release since the glucocorticoid, dexamethasone inhibited both effects. Thus, LPS (4 mg kg⁻¹) increased the plasma levels of nitrite/nitrate from 14 ± 1 to 84 ± 7 µM within 3 h and this rise was inhibited to 35 ± 1 µM by dexamethasone. Levels of 6-keto PGF_{1α} in the plasma were below the detection limit of the assay (< 0.2 ng ml⁻¹). However, 3 h after the injection of LPS these levels rose to 2.6 ± 0.2 ng ml⁻¹ and to 0.7 ± 0.01 ng ml⁻¹ after LPS in rats that received dexamethasone.

2 The induced enzymes were inhibited *in vivo* with selective COX and NOS inhibitors. Furthermore, NOS inhibitors, that did not affect COX activity *in vitro* markedly suppressed PG production in the LPS-treated animals. For instance, the LPS-induced increase in plasma nitrite/nitrate and 6-keto PGF_{1α} at 3 h was decreased to 18 ± 2 µM and 0.5 ± 0.02 ng ml⁻¹, 23 ± 1 µM and 0.7 ± 0.01 ng ml⁻¹, 29 ± 2 µM and 1 ± 0.01 ng ml⁻¹ in rats treated with LPS in the presence of the NOS inhibitors N^G-monomethyl-L-arginine, N^G-nitro arginine methyl ester and aminoguanidine, respectively.

3 The intravenous infusion of the NO donors sodium nitroprusside (SNP) or glyceryl trinitrate (GTN) increased prostaglandin production in normal animals (for instance urinary PGE₂ excretion was increased from 96 ± 10 to 576 ± 12 pg min⁻¹ and 400 ± 24 pg min⁻¹ in the presence of GTN or SNP respectively).

4 Proteinuria was measured in order to evaluate the roles of NO and PG in renal damage associated with the *in vivo* injection of LPS. Interestingly, dexamethasone and the NOS inhibitors attenuated proteinuria in the LPS-treated rats. The COX inhibitors had no effect. It therefore appears that NO and not PG contributes to the LPS-induced renal damage; these findings support the potential use of NOS inhibitors in the treatment of renal inflammation.

5 This study demonstrates the regulatory contribution of NO on the *in vivo* production of prostanoids and suggests that in inflammatory diseases that are driven by both NO and the prostaglandins, NOS inhibitors may act to reduce inflammation by the dual inhibition of cytotoxic NO and pro-inflammatory PG.

Keywords: Nitric oxide synthase inhibitors; cyclo-oxygenase; prostaglandins; *E.coli* lipopolysaccharide; nitric oxide

Introduction

Nitric oxide (NO) is derived from L-arginine (L-Arg) by the enzyme nitric oxide synthase (NOS) whereas cyclo-oxygenase (COX) converts arachidonic acid to prostaglandins (PG). There are two major forms of NOS and COX. The constitutively expressed isoforms of these enzymes are found in numerous cell types in the absence of immunostimulation (see Moncada *et al.*, 1991 and DeWitt 1991 for reviews). Production of NO and prostaglandins from the constitutive isoforms is important for the normal physiological function of various systems. The inducible isoforms (iNOS and COX-2) are not normally expressed, but are induced following appropriate stimulation with pro-inflammatory agents such as *E.coli* lipopolysaccharide (LPS) (Fu *et al.*, 1990; Masferrer *et al.*, 1990; 1992; Moncada *et al.*, 1991). Anti-inflammatory steroids, including dexamethasone, inhibit the induction of iNOS and COX-2 *in vitro* and *in vivo* with no apparent effect on the constitutive forms of the enzymes (Masferrer *et al.*, 1990; 1992; Radomski *et al.*, 1990; Moncada *et al.*, 1991). Inhibition of NO in LPS-induced macrophages *in vitro* results in an attenuation of PG release from these cells (Salvemini *et al.*, 1993). This action of NO on the COX pathway has been confirmed as well in other cell systems including hypothalamic slices (Rettori *et al.*, 1992), smooth muscle cells (Inoue *et al.*, 1993), islet cells (Corbett *et al.*,

1993) and in an intact organ system (Salvemini *et al.*, 1994).

To date, most of the effects of NO on COX activity and hence PG production have been assessed in cells or enzyme preparations. It is not yet known whether the COX pathway is modulated by NO *in vivo*. We have addressed this question in our study in order to unravel the therapeutic implications of our *in vitro* discovery. *In vivo* administration of LPS in rats induces iNOS resulting in increased production of NO₂⁻, a breakdown product of NO (see Moncada *et al.*, 1991; Moncada & Higgs, 1993 for reviews). Furthermore, LPS injection in rats has been shown to cause marked *in vivo* stimulation of arachidonic acid metabolism resulting in a profound increase in plasma and urinary prostaglandin (see Flynn, 1985 for review). We have therefore used this *in vivo* model to evaluate the contributions of NO on the exaggerated production of PG evoked by bolus injection of LPS in conscious, restrained rats.

Methods

In vivo measurements

Male Sprague Dawley rats (300–350 g) were purchased from Charles River (Wilmington, MA, U.S.A.) and used through-

¹ Author for correspondence.

out these studies. All animal protocols were approved by the Institutional Animal Care and Use Committee. Animals were anaesthetized with methoxyflurane before surgical preparation. Polyethylene catheters (PE50), were inserted into a femoral artery and vein. The urinary bladder was cannulated with a flared PE90 polyethylene catheter. The animals were placed into individual restraining cages and allowed to regain consciousness. Mean arterial blood pressure and heart rate were measured continuously via the femoral artery with a pressure transducer (type 041-500-503, Cobe, Lakewood, CO, U.S.A.) connected to a Grass Model 7E polygraph. Mean arterial pressure and heart rate were recorded at 5 min intervals on a computerized data acquisition system.

Drug administration and protocols

The lowest dose of LPS that promoted a maximal iNOS induction with minimal cardiovascular changes in our conscious rats was found to be 4 mg kg^{-1} . Higher doses were not used since they promoted severe deleterious effects that hindered the collection of blood and urine samples. Upon completion of the surgical procedure, cardiovascular parameters were allowed to stabilise for 30 min. Rats then received a bolus intravenous injection of 4 mg kg^{-1} LPS (serotype 0111:B4) dissolved in saline. Haemodynamic parameters were monitored for a subsequent 5 h. All drug treatments began 30 min prior to the injection of LPS. Urine was collected during this time frame and a blood sample ($500 \mu\text{l}$) was taken from the femoral artery immediately before and after drug administration. These time points are referred to as T_0 ; the amount of NOS and COX products obtained at T_0 reflect basal levels. Subsequent blood samples were taken every hour for 5 h after LPS or saline; 60 min urine volumes were collected for the duration of the experiment.

The non-selective cNOS and iNOS inhibitors, N^G -nitro-L-arginine methyl ester (L-NAME, $0.1 \text{ mg kg}^{-1} \text{ min}^{-1}$) or N^G -monomethyl-L-arginine (L-NMMA, $1 \text{ mg kg}^{-1} \text{ min}^{-1}$) or the more selective iNOS inhibitor, aminoguanidine (AG, $1 \text{ mg kg}^{-1} \text{ min}^{-1}$) were infused for the entire duration of the experiment via the femoral vein. Control rats received an infusion of 0.9% sodium chloride via the femoral vein ($0.07 \text{ ml kg}^{-1} \text{ min}^{-1}$) for the entire duration of the study. Indomethacin (or an equivalent volume of the vehicle; phosphate buffered saline, 1% Tween 80, pH 7.4) or dexamethasone were given intraperitoneally at a dose of 3 mg kg^{-1} or 4 mg kg^{-1} respectively. The selective COX-2 inhibitor, NS398 (Futaki *et al.*, 1993; Masferrer *et al.*, 1994b) was given by gavage (10 mg kg^{-1}).

In some experiments, rats were treated with LPS for 4 h and then received an intravenous bolus injection of AG (1 mg kg^{-1}), dexamethasone (3 mg kg^{-1}) or an equivalent volume of saline (0.5 ml). Blood samples were taken immediately before and at 1 h after the injection of saline, AG or dexamethasone for the determination of plasma NO_2^- and 6-keto $\text{PGF}_{1\alpha}$.

In order to evaluate the role of exogenous nitric oxide on *in vivo* prostaglandin formation two nitric oxide donors namely sodium nitroprusside (SNP) and glyceryl trinitrate (GTN) were used. The effects of verapamil, a vasodilator which does not release NO was also used. SNP ($0.125 \text{ mg kg}^{-1} \text{ min}^{-1}$), GTN or verapamil ($0.25 \text{ mg kg}^{-1} \text{ min}^{-1}$) were infused for a total period of 1 h; blood samples were taken before and after drug administration and urine was collected throughout the 1 h period of infusion.

Mediators measured to assess the presence of the NOS and COX systems

The production of nitrite/nitrate ($\text{NO}_2^-/\text{NO}_3^-$), breakdown products of NO, or PGE_2 and 6-keto $\text{PGF}_{1\alpha}$ mediators released after activation of COX, were used, respectively as markers for the presence of the L-arginine to NO pathway and the COX pathway. NO stimulates the soluble guanylate

cyclase leading to increased excretion of guanosine 3':5'-cyclic monophosphate (cyclic GMP) which can be detected in the urine (Wilkins *et al.*, 1990). Therefore, urinary cyclic GMP levels were also measured as an index of the activation of the L-arginine to nitric oxide pathway. Total excretion of $\text{NO}_2^-/\text{NO}_3^-$, cyclic GMP and prostanooids in the urine was calculated, based on the concentration and the urine volume so as to take into account any changes in urinary output.

Nitrite/nitrate determination

Arterial blood was collected in Microtainer tubes containing lithium heparin (Becton Dickinson, Rutherford, NJ, U.S.A.) and centrifuged to collect plasma samples. Plasma samples were passed through an Ultra-MC filter (10,000 MWCO, Millipore, Bedford, MA, U.S.A.). NO reacts with oxygen to yield nitrate and nitrite. Nitrate in plasma aliquots ($5 \mu\text{l}$) was converted to nitrite by the addition of nitrate reductase (14 mu) and the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH, 1 nmol) with incubation for 10 min at room temperature. The reaction was terminated by dilution with water and addition of the diaminonaphthalene reagent (Misko *et al.*, 1993b). Nitrite concentrations in these samples were determined fluorimetrically (Misko *et al.*, 1993b) with sodium nitrite used as a standard. This procedure was also used to determine the levels of $\text{NO}_2^-/\text{NO}_3^-$ excreted in the urine. All determinations were performed in duplicate. Results are expressed as plasma or urinary $\text{NO}_2^-/\text{NO}_3^-$ levels.

PGE_2 , 6-keto $\text{PGF}_{1\alpha}$, cyclic GMP and protein determinations

6-keto $\text{PGF}_{1\alpha}$ was measured in the unextracted plasma by ELISA (Cayman Chemicals, Ann Arbor, MI, U.S.A.). (Fletcher *et al.*, 1981). Urinary levels of 6-keto $\text{PGF}_{1\alpha}$ and PGE_2 were determined by specific ELISAs. Concentrations of cyclic GMP in urine were measured by radioimmunoassay as described previously (Wilkins *et al.*, 1990). Urinary protein excretion was measured by the Bradford assay (Bio-Rad Laboratories, CA, U.S.A.). Each experiment was performed in duplicate.

Preparation of human recombinant (r) COX enzymes

Insect cells expressing human COX-1 or COX-2 were homogenized as previously described (Masferrer *et al.*, 1994b) and membranes were incubated with arachidonic acid ($10 \mu\text{M}$) for 15 min. COX activity was determined by measuring PGE_2 by ELISA, expressed as $\text{ng PGE}_2 \mu\text{g}^{-1} \text{ protein min}^{-1}$. Drugs

Figure 1 Effects of cyclooxygenase inhibitors (indomethacin and NS398) and dexamethasone on LPS-induced increases in plasma 6-keto prostaglandin $\text{F}_{1\alpha}$ (6-keto $\text{PGF}_{1\alpha}$) levels. *E. coli* lipopolysaccharide (LPS, 4 mg kg^{-1} , i.v., $n = 11$) caused a time-dependent increase in the levels of 6-keto $\text{PGF}_{1\alpha}$ (●) and this was attenuated by indomethacin (○, $n = 6$), NS398 (△, $n = 4$) and dexamethasone (□, $n = 6$). Each point is the mean $\pm$ s.e.mean for n experiments.

were preincubated with homogenates for 10 min before the addition of arachidonic acid.

Drugs and chemicals

Reagents were obtained from Sigma Chemical Co (St Louis, MO, U.S.A.). NS398 [N-(2-cyclohexyloxy-4-nitrophenyl)-methanesulphonamide] was synthesized by J. Rogier and J. Talley, Monsanto Corporate Research as previously described (Futaki *et al.*, 1993). Indomethacin and NS398 were dissolved in phosphate-buffered saline, pH 7.4/1% (v/v) Tween-80. All other compounds were dissolved in saline.

Statistical analysis

Statistical significance of difference between groups was determined by a two-way analysis of variance (ANOVA) followed by Student's unpaired *t* test. A probability (*P*) value of 0.05 or less was taken to indicate statistical significance. Results are expressed as mean $\pm$ s.e.mean for (*n*) experiments.

Results

Induction of the COX pathway by LPS: effects of COX inhibitors and dexamethasone

An increase in plasma and urinary 6-keto PGF_{1 α} (Figures 1 and 2b) and urinary PGE₂ (Figure 2a) was observed 1 h post-LPS injection and reached a peak by 2 h (Figures 1 and 2). The non-selective COX-1/COX-2 inhibitor, indomethacin (3 mg kg⁻¹, i.p., *n* = 6), blocked the rise in these arachidonic acid metabolites at all time points (Figures 1 and 2) whereas the inhibitory effects of dexamethasone (4 mg kg⁻¹ i.p., *n* = 6) or of the COX-2 selective inhibitor NS398 (Masferrer *et al.*, 1994b; 10 mg kg⁻¹ p.o., *n* = 4) were observed 2 h post-LPS and continued to the end of the experiment (Figures 1 and 2).

Effects of NOS inhibitors on prostanoid production

The basal plasma nitrite, urinary nitrite and cyclic GMP levels were respectively, 14 ± 1 μ M, 1.25 ± 0.1 nmol min⁻¹ and 51 ± 8 pmol min⁻¹ (*n* = 11). L-NMMA or L-NAME slightly but significantly reduced these levels (Table 1). Thereafter, plasma NO₂⁻/NO₃⁻ (Table 1), urinary NO₂⁻/NO₃⁻ and cyclic GMP levels (Table 2A and B) started to increase 2 h post-LPS injection and by 5 h the values attained were, respectively, at least 18, 33 and 47 fold higher than basal (*n* = 11). Intravenous infusion of either L-NMMA (1 mg kg⁻¹ min⁻¹, *n* = 6), L-NAME (0.1 mg kg⁻¹ min⁻¹, *n* = 7), AG (1 mg kg⁻¹ min⁻¹, *n* = 8) or dexamethasone (4 mg kg⁻¹ i.p., *n* = 6) blocked the LPS-induced rise in NO₂⁻/NO₃⁻ and cyclic GMP (Tables 1 and 2). Inhibition of NO formation by the NOS inhibitors at these time points, resulted in a marked

inhibition of PG in plasma (Figure 3) and urine (Figure 4a and b). In some experiments, rats were treated first with LPS (so as to induce the NOS and COX systems) for 4 h and then received an intravenous bolus injection of AG (1 mg kg⁻¹), dexamethasone (3 mg kg⁻¹) or an equivalent volume of saline (0.5 ml). Blood samples were taken immediately before and at 1 h after the injection of saline, AG or dexamethasone for the determination of plasma NO₂⁻/NO₃⁻ and 6-keto PGF_{1 α} . Table 3 shows that AG caused a fall in the levels of NO₂⁻/NO₃⁻ (from 150 ± 7 μ M before AG to 60 ± 7 μ M after AG, *n* = 4, *P* < 0.05) which was associated with a fall in 6-keto PGF_{1 α} (from 2.7 ± 0.2 ng ml⁻¹ before AG to 0.7 ± 0.01 ng ml⁻¹ after AG, *n* = 4, *P* < 0.05). Dexamethasone had no effect (Table 3).

Figure 2 Effects of cyclo-oxygenase inhibitors and dexamethasone on *E. coli* lipopolysaccharide (LPS)-induced increases in urinary prostaglandin E₂ (PGE₂) and 6-keto PGF_{1 α} levels. LPS (4 mg kg⁻¹, i.v.) caused a time-dependent increase (●) in the urinary levels of PGE₂ (a) and 6-keto PGF_{1 α} (b) and this was abolished by indomethacin (○, *n* = 6), NS398 (Δ, *n* = 4) or dexamethasone (□, *n* = 6) (a and b). Each point is the mean $\pm$ s.e.mean for *n* experiments.

Table 1 *E. coli* lipopolysaccharide (LPS) increases the plasma levels in nitrite/nitrate: effects of NO synthase (NOS) inhibitors (L-NMMA, L-NAME and AG), cyclo-oxygenase inhibitors (indomethacin, NS398) and dexamethasone

Drugs	Time (h) after LPS					
	0	1	2	3	4	5
None	14 $\pm$ 1	13 $\pm$ 1	60 $\pm$ 5	84 $\pm$ 7	160 $\pm$ 12	256 $\pm$ 9
L-NMMA	7 $\pm$ 0.5*	6 $\pm$ 1*	15 $\pm$ 3*	18 $\pm$ 2**	16 $\pm$ 4**	22 $\pm$ 4**
L-NAME	7 $\pm$ 0.3*	7 $\pm$ 1*	10 $\pm$ 2*	23 $\pm$ 1**	36 $\pm$ 3**	40 $\pm$ 3**
AG	23 $\pm$ 4	23 $\pm$ 3	20 $\pm$ 5*	29 $\pm$ 2**	26 $\pm$ 3**	24 $\pm$ 2**
Indo	12 $\pm$ 1	13 $\pm$ 1	50 $\pm$ 1	75 $\pm$ 5	130 $\pm$ 7	213 $\pm$ 13
NS398	19 $\pm$ 1	11 $\pm$ 2	59 $\pm$ 5	76 $\pm$ 6	180 $\pm$ 5	215 $\pm$ 40
Dex	14 $\pm$ 1	12 $\pm$ 1	20 $\pm$ 1*	35 $\pm$ 1**	33 $\pm$ 1**	33 $\pm$ 2**

The NOS inhibitors or dexamethasone (Dex) prevented the increase in the levels of NO₂⁻/NO₃⁻ (μ M). Indomethacin (Indo) or NS398 were ineffective. **P* < 0.05 and ***P* < 0.01 when compared to the values obtained with LPS alone. AG, aminoguanidine; L-NMMA: N^G-monomethyl-L-arginine; L-NAME: N^G-nitro-L-arginine methyl ester.

Table 2 Effects of nitric oxide synthase inhibitors, cyclo-oxygenase inhibitors and dexamethasone (Dex) on *E. coli* lipopolysaccharide (LPS)-induced increases in urinary nitrite/nitrate (A, nmol min⁻¹) and cyclic GMP (B, pmol min⁻¹) output in rats

		Time (h) after LPS					
A							
Drugs	0	1	2	3	4	5	
None	125 ± 0.1	2.27 ± 0.5	13 ± 1.2	55 ± 8	40 ± 5	42 ± 6	
L-NMMA	0 ± 0*	0 ± 0*	2 ± 0.1*	7 ± 0.5**	7 ± 1**	6 ± 2**	
L-NAME	0.7 ± 0.01*	1 ± 0.1*	3 ± 0.03*	9 ± 0.2**	10 ± 2**	6 ± 1**	
AG	2 ± 0.2	2 ± 1	5 ± 1	7 ± 2**	5 ± 1**	4 ± 0.5**	
Indo	2 ± 1	3 ± 1	10 ± 1	50 ± 3	33 ± 4	40 ± 5	
NS398	2 ± 1	5 ± 2	12 ± 4	60 ± 5	55 ± 3	40 ± 10	
Dex	1 ± 0.1	3 ± 1	4 ± 0.5*	6 ± 2**	7 ± 1**	6 ± 0.5**	
B							
None	51 ± 8	49 ± 7	669 ± 180	3568 ± 200	2976 ± 93	2407 ± 48	
L-NMMA	20 ± 5*	20 ± 7*	70 ± 4**	90 ± 3**	90 ± 10**	70 ± 5**	
L-NAME	22 ± 5*	21 ± 1*	80 ± 13**	101 ± 8**	99 ± 12**	74 ± 21**	
AG	45 ± 8	59 ± 9	96 ± 10*	96 ± 6**	76 ± 6**	73 ± 25**	
Indo	53 ± 6	48 ± 7	492 ± 115	2383 ± 700	2000 ± 230	2426 ± 64	
NS398	55 ± 9	50 ± 8	500 ± 100	3900 ± 300	3000 ± 200	2000 ± 200	
Dex	51 ± 10	58 ± 10	114 ± 29*	138 ± 31**	136 ± 25**	143 ± 29**	

* $P < 0.05$ and ** $P < 0.01$ when compared to values obtained in the absence of drugs. For abbreviations, see Table 1

Figure 3 Effects of nitric oxide synthase inhibitors (L-NMMA, L-NAME and AG) on *E. coli* lipopolysaccharide (LPS)-induced increases in plasma 6-keto prostaglandins F_{1α} (6-keto PGF_{1α}) levels. Intravenous infusion of N^G-monomethyl-L-arginine (L-NMMA, ○, 1 mg kg⁻¹ min⁻¹, $n = 6$), N^G-nitro-L-arginine methyl ester (L-NAME, △, 0.1 mg kg⁻¹ min⁻¹, $n = 7$) or aminoguanidine (AG, □, 1 mg kg⁻¹, $n = 8$) attenuated the increase in 6-keto PGF_{1α} following LPS administration (●). Each point is the mean ± s.e.mean for n experiments.

The NOS inhibitors, L-NMMA, L-NAME or aminoguanidine (1–300 μM, $n = 6$) failed to inhibit human recombinant COX-1 and COX-2 activity directly (Figure 5a and b). Similar results were obtained with murine recombinant COX-1 and COX-2 (data not shown). Indomethacin inhibited COX-1 (IC₅₀ = 0.058 μM, $n = 10$; Figure 5a) and COX-2 (IC₅₀ = 1.1 μM, $n = 10$; Figure 5b), whereas NS398 selectively inhibited COX-2 (IC₅₀ = 0.1 μM, $n = 10$; Figure 5b), with no effect on COX-1 (Figure 5a). The observation that the IC₅₀ of indomethacin for COX-2 is higher than for COX-1 has been reported by others (Meade *et al.*, 1993; Gierse *et al.*, 1994). The assumption is (although not proven) that this is due to the intrinsic ability of indomethacin to bind differently the active site of COX-1 versus COX-2. In contrast to the effects of the NOS inhibitors, *in vivo* administration of indomethacin or NS398 had no effect on the LPS-induced increase in nitrite or cyclic GMP production (Tables 1 and 2).

Release of 6-keto PGF_{1α} in plasma by the administration of NO-donors in normal rats

The intravenous infusion (for 1 h) of glyceryl trinitrate (0.25 mg kg⁻¹ min⁻¹, $n = 6$) or sodium nitroprusside

Figure 4 Effects of NO synthase (NOS) inhibitors on *E. coli* lipopolysaccharide (LPS)-induced increases in urinary prostaglandin E₂ (PGE₂) and 6-keto PGF_{1α} levels. Intravenous infusion of N^G-monomethyl-L-arginine (○, $n = 6$), N^G-nitro-L-arginine methyl ester (△, $n = 7$) or aminoguanidine (□, $n = 8$) attenuated the rise (●) in urinary output of PGE₂ (a) and 6-keto PGF_{1α} (b). Each point is the mean ± s.e.mean for n experiments.

(0.125 mg kg⁻¹ min⁻¹, $n = 6$) caused a 14 fold increase in the plasma levels of NO₂⁻/NO₃⁻ (from 14 ± 1 μM to 200 ± 20 μM and 175 ± 15 μM respectively). Levels of 6-keto PGF_{1α} in the plasma were below the detection limit of the assay (< 0.2 ng ml⁻¹). These increased respectively to 2.5 ± 0.3 ng ml⁻¹ and 1.7 ± 0.5 ng ml⁻¹ following the 1 h infusion with either GTN or SNP. Urinary PGE₂ excretion was increased from 96 ± 10 to 576 ± 12 pg min⁻¹ and to

Table 3 The effects of aminoguanidine (AG) or dexamethasone (Dex) on plasma $\text{NO}_2^-/\text{NO}_3^-$ (μM) and 6-keto $\text{PGF}_{1\alpha}$ (ng ml^{-1}) at 4 h after *E. coli* lipopolysaccharide (LPS) injection

	Before bolus injection of the drug	1 h after bolus injection of the drug
None		
NO_2^-	140 ± 12	300 ± 15
6-keto $\text{PGF}_{1\alpha}$	2.5 ± 0.1	3 ± 0.2
AG		
NO_2^-	$150 \pm 7^*$	$60 \pm 7^*$
6-keto $\text{PGF}_{1\alpha}$	$2.7 \pm 0.2^*$	$0.7 \pm 0.01^*$
Dex		
NO_2^-	135 ± 9	285 ± 10
6-keto $\text{PGF}_{1\alpha}$	2 ± 0.1	2.2 ± 0.5

Rats were treated with LPS for 4 h and then received an intravenous bolus injection of AG, Dex or an equivalent volume of saline (0.5 ml). Blood samples were taken immediately before and at 1 h after treatment. AG caused an immediate fall in the levels of $\text{NO}_2^-/\text{NO}_3^-$ and this was associated with an immediate fall in 6-keto $\text{PGF}_{1\alpha}$. Dex had no effect. Each point is the mean $\pm$ s.e.mean for 4 experiments. * $P < 0.05$ when compared to values obtained before the injection of saline, AG or Dex.

$400 \pm 24 \text{ pg min}^{-1}$ in the presence of GTN or SNP respectively ($n = 6$). Verapamil when infused for 1 h at a dose ($0.25 \text{ mg kg}^{-1} \text{ min}^{-1}$, $n = 8$) that elicited a similar fall in blood pressure to the one evoked by the NO-donors, failed to increase the levels of $\text{NO}_2^-/\text{NO}_3^-$ or PG (data not shown).

Mean arterial pressure decreased from a basal level of $120 \pm 2 \text{ mmHg}$ to $93 \pm 2 \text{ mmHg}$, $80 \pm 4 \text{ mmHg}$ and $61 \pm 4 \text{ mmHg}$ following the 1 h period of infusion with GTN, SNP or verapamil respectively.

Effects of NOS or COX inhibitors and dexamethasone on proteinuria

Urinary protein excretion was observed 2 h post-LPS injection, reached a peak by 3 h and remained constant for the remaining of the experiment (Figure 6). Proteinuria was inhibited by at least 50% by aminoguanidine (Figure 6a) or dexamethasone (Figure 6b) and to a lesser extent by L-NMMA or NO_2Arg (Figure 6a) but was not affected by indomethacin or NS398 (Figure 6b).

Haemodynamic effect

Inhibition of cNOS by non-selective cNOS/iNOS inhibitors such as L-NMMA or L-NAME has been shown to increase blood pressure in a variety of animal species (see Moncada *et al.*, 1991; Moncada & Higgs, 1993 for reviews). This phenomenon is not observed with selective iNOS inhibitors such as aminoguanidine (Misko *et al.*, 1993a; Griffith *et al.*, 1993) or with steroids such as dexamethasone (see Moncada *et al.*, 1991; Moncada & Higgs, 1993 for reviews). Mean arterial blood pressure (MAP) and heart rate in conscious normotensive restrained rats was $113 \pm 2 \text{ mmHg}$ and $410 \pm 2 \text{ b.p.m.}$ ($n = 16$). In normotensive rats and at the doses used throughout our experiments, L-NMMA or L-NAME increased MAP by 35 mmHg within 30 min post infusion; the increase in MAP elicited by these agents remained at that level for the duration of the experiment (6 h). Aminoguanidine or dexamethasone did not increase MAP in normotensive animals ($n = 6$, data not shown), consistent with their selectivity for iNOS.

LPS injection caused a slight fall in mean arterial pressure within approximately 90 min which returned to basal levels by 2 h (Table 4). In contrast to their lack of effect on MAP

Figure 5 Effects of cyclo-oxygenase (COX) inhibitors and NO synthase (NOS) inhibitors on human recombinant COX-1 and COX-2 (hrec COX-1 and hrec COX-2) activity. The non-selective COX-1 and COX-2 inhibitor, indomethacin, blocked the activity of both hrec COX-1 and hrec COX-2 (●, $n = 10$) whereas the selective COX-2 inhibitor NS398 affected only hrec COX-2 activity (▲, $n = 10$) (a and b). The NOS inhibitors, N^G -monomethyl-L-arginine (○, $n = 6$), N^G -nitro-L-arginine methyl ester (△, $n = 6$) or aminoguanidine (□, $n = 6$) had no effect (a and b). Results are expressed as prostaglandin E_2 (PGE_2) release (% of control).

in normotensive rats, aminoguanidine or dexamethasone increased MAP in the LPS-treated rats; this effect was observed within 2 h after the injection of LPS and for the remainder of the experiment (Table 4). Indomethacin or NS398 did not increase BP in normotensive rats or rats treated with LPS (Table 4).

Discussion

We recently demonstrated *in vitro* that NO can activate COX resulting in an increase in the production of prostaglandins. In this study we demonstrate a similar relationship between NO and COX *in vivo*.

LPS caused an increase in plasma 6-keto $\text{PGF}_{1\alpha}$ and urinary excretion of 6-keto $\text{PGF}_{1\alpha}$ and PGE_2 over time although the peak urinary excretion of PGE_2 occurred before (1 h post LPS) the one obtained for 6-keto $\text{PGF}_{1\alpha}$ (2 h post LPS). Different cell types which respond differently to LPS or a difference in the kinetics of their isomerases may account for this observation. For purposes of clarity we will refer to the release of prostanoids (6-keto $\text{PGF}_{1\alpha}$ and PGE_2) that occurred within 1 h post LPS as 'acute release' and the one that occurred within 2 h post LPS as 'delayed release'. Acute release of PG was inhibited by indomethacin, but not by NS398 a selective COX-2 inhibitor (Masferrer *et al.*, 1994b) or dexamethasone suggesting that constitutive COX-1 is responsible for the acute release of PG elicited by LPS; constitutive COX-1 activation may result from the LPS-promoted

release of vasoactive substances such as kinins (Parratt, 1983; Wilson *et al.*, 1989). NS398 or dexamethasone inhibited the delayed release of prostanoid indicating that induced COX-2 is responsible for the delayed release of PG. This is substantiated by the findings that within 2 h after LPS injection in rats and mice COX-2 mRNA and protein was expressed in various organs (Seibert *et al.*, 1994) and inflammatory cells (Masferrer *et al.*, 1990; 1994a).

Besides activating the COX pathway, LPS activated the NOS system within a similar time frame. Release of NO (as assessed by measurements of $\text{NO}_2^-/\text{NO}_3^-$ and cyclic GMP) observed at 1 h post-LPS injection was aminoguanidine and dexamethasone-insensitive, but inhibitable by the non-

selective cNOS/iNOS inhibitors L-NMMA and L-NAME. This indicates that cNOS accounts for NO release during the acute phase and iNOS accounts for NO release during the later phase of endotoxin administration. This is supported by the demonstration that LPS releases NO from cNOS within minutes *in vitro* and *in vivo* (Salvemini *et al.*, 1990; Thiermermann & Vane, 1990; Fleming *et al.*, 1992) and that within 2 h post injection it induces iNOS mRNA and protein in various organs (Salter *et al.*, 1991; Liu *et al.*, 1993).

LPS stimulates the release of a variety of mediators (noradrenaline, angiotensin II, 5-hydroxytryptamine, thromboxane) with vasoconstrictor capacities that would oppose the vasodilator effects of NO (see Moncada *et al.*, 1991, for review). Therefore, a balance between vasoconstrictor/vasodilator mediators is involved in the maintenance of the blood pressure level. This may explain why LPS at the dose used in our study did not (as intended) cause measurable changes in blood pressure yet induced NOS. Nevertheless, the effects of NO on the modulation of blood vessel tone was detected when iNOS activity (by AG) or iNOS induction (by dexamethasone) were inhibited. Thus, AG or Dex increased blood pressure 2/3 h post LPS injection whereas they had no effect in saline-treated animals.

What is the consequence of NO inhibition *in vivo* on the production of PG? Using *in vitro* systems, we and others have reported that inhibition of NO has inhibitory effects on PG release, whereas addition of exogenous NO augments PG release (Rettori *et al.*, 1992; Salvemini *et al.*, 1993; Corbett *et al.*, 1993; Inoue *et al.*, 1993; Salvemini *et al.*, 1994). Here we have shown that when the increased endogenous production of NO was blocked by the NOS inhibitors at the time point of iNOS induction (2 h), the increase in plasma and urinary production of prostanoids was inhibited by at least 50%. This suggests that induction of iNOS release enough NO to modulate the COX pathway. This was demonstrated further by showing that removal of NO by bolus injection of AG 4 h after LPS (a time point when iNOS was induced) resulted in a concomitant decrease in PG production. Under the same experimental conditions, dexamethasone failed to alter $\text{NO}_2^-/\text{NO}_3^-$ and PG formation consistent with the fact that once iNOS is induced, NO release cannot be affected by this steroid (Wright *et al.*, 1992; Paya *et al.*, 1993). The ability of the NOS inhibitors to inhibit PG production was not due to direct inhibition of COX enzymes for they failed to affect the activity of human recombinant COX-1 or COX-2.

The effects of endogenously produced NO on prostaglandin formation were mimicked by the exogenous *in vivo* administration of NO. Thus, in normal rats, release of $\text{NO}_2^-/\text{NO}_3^-$ by glyceryl trinitrate or sodium nitroprusside was accompanied by an increase in the plasma levels of 6-keto $\text{PGF}_{1\alpha}$. PG release elicited by the NO-donor must be reflecting COX-1 activation because COX-1 and not COX-2 mRNA and protein is found in organs from normal animals

Figure 6 Effects of drugs on the proteinuria elicited by *E. coli* lipopolysaccharide (LPS). The increase in urinary excretion of protein elicited by LPS (●, $n = 11$) was attenuated by N^G -monomethyl-L-arginine (□, $n = 6$), N^G -nitro-L-arginine methyl ester (○, $n = 7$) or aminoguanidine (■, $n = 8$) (a) and dexamethasone ($n = 6$) (b) but not by indomethacin (○, $n = 6$) or NS398 (△, $n = 4$) (b). Results are expressed as $\mu\text{g protein min}^{-1}$ for n experiments.

Table 4 Blood pressure (mmHg) determinations in conscious, restrained Sprague Dawley rats treated with *E. coli* lipopolysaccharide (LPS, 4 mg kg^{-1} , i.v.) in the absence or presence of various drugs

Time (min)	Drugs						
	None	L-NAME	L-NMMA	AG	Dex	Indo	NS398
-30	113 ± 2	112 ± 2	113 ± 3	112 ± 3	113 ± 3	112 ± 2	112 ± 1
0	116 ± 2	127 ± 7*	127 ± 7*	116 ± 3	116 ± 3	111 ± 2	110 ± 2
30	114 ± 2	152 ± 4*	152 ± 4*	115 ± 2	112 ± 2	113 ± 4	110 ± 2
60	113 ± 1	146 ± 4*	146 ± 4*	113 ± 3	112 ± 3	115 ± 5	117 ± 1
90	106 ± 2	145 ± 3*	145 ± 3*	129 ± 2*	126 ± 3*	102 ± 5	103 ± 2
120	117 ± 2	144 ± 4*	144 ± 4*	140 ± 1*	136 ± 3*	121 ± 4	120 ± 2
150	120 ± 2	144 ± 4*	144 ± 4*	141 ± 2*	138 ± 2*	121 ± 5	121 ± 2
180	120 ± 2	148 ± 4*	148 ± 4*	140 ± 2*	133 ± 4*	124 ± 4	121 ± 2
210	119 ± 2	146 ± 4*	146 ± 4*	145 ± 1*	130 ± 2*	118 ± 5	119 ± 3
240	115 ± 3	146 ± 6*	146 ± 6*	140 ± 2*	138 ± 2*	112 ± 3	118 ± 2

Drugs were administered 30 min before LPS injection (-30). * $P < 0.05$ when compared to values obtained at the corresponding time point for the none group. Each point is the mean ± s.e.mean for 7–11 experiments. See Table 1 for abbreviations.

(Seibert *et al.*, 1994). In addition, PG release by the NO-donors was not a consequence of changes in blood pressure for verapamil (a non NO-donor) when used at a dose which caused a fall in blood pressure similar to the one obtained with GTN or SNP, did not increase plasma PG.

Appearance of protein in the urine following LPS injection is an index of renal damage elicited by endotoxin (see Schlag & Redl, 1987, for review). Figure 6 shows that the time course for proteinuria was similar to the one obtained for NO and PG. Aminoguanidine or dexamethasone, but not the COX inhibitors abrogated the development of proteinuria indicating that NO release from iNOS is responsible at least in part for renal damage. These findings will allow us to evaluate the potential use of iNOS inhibitors in the management of renal damage. A role for iNOS in mediating glomerular damage in models of renal inflammation has also been reported (Cattell *et al.*, 1990; Cook & Sullivan, 1991; Jansen *et al.*, 1992). A weaker protective effect was observed with the non-selective cNOS/iNOS inhibitor L-NMMA or L-NAME. Since the release of NO from cNOS is important for maintaining adequate perfusion to a variety of vital organs including the kidney (see Luscher & Bock, 1991; King & Brenner, 1991, for reviews) it is possible that inhibition of

cNOS in an already compromised organ (kidney inflammation elicited by LPS) may counteract the beneficial effects that would result if only iNOS were inhibited. The recent discovery that the COX pathway is modulated by NO, indicates that inhibition of NOS activity may remove the drive that NO has on COX, thereby lowering the amounts of pro-inflammatory PG; dual inhibition of the release of pro-inflammatory NO and PG could therefore contribute to the anti-inflammatory properties of NOS inhibitors. Possible mechanisms by which NO augments PG release include, but is not restricted to, direct activation of the COX enzymes (Salvemini *et al.*, 1993; Hajjar *et al.*, 1994) and inhibition of the autoinactivation of the COX enzymes, a phenomenon associated with the production of free radicals (Egan *et al.*, 1976).

The effects of the iNOS inhibitors may provide a therapeutic advantage over steroids in that they may provide anti-inflammatory effects without the mechanism-based side effects described with chronic use of steroids.

We thank Dr Allen Nickols for the review of this manuscript and Carol Koboldt for her help with the recombinant COX experiments.

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(Received October 31, 1994

Revised November 14, 1994

Accepted November 29, 1994)