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Characterization of the induction of nitric oxide synthase and cyclo-oxygenase in rat aorta in organ culture

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1 Within vessels, the formation of nitric oxide (NO) or prostaglandins is normally catalysed in the endothelium by constitutive isoforms of NO synthase (eNOS) and cyclo-oxygenase (COX-1), respectively. However, during inflammatory conditions, the underlying smooth muscle acquires the ability to release NO and prostaglandins after the expression of inducible isoforms of NOS (iNOS) and COX (COX-2). The co-induction of iNOS and COX-2 has been studied over 24 h in isolated vascular smooth muscle cells *in vitro*. However, due to the limitation of using cultured cells, the relationship between the activities of iNOS and COX over longer periods has not been addressed. Moreover, the relative contribution of the endothelium to the production of NO and prostaglandins under inflammatory conditions is not completely understood.

2 Here using an organ culture system, we have determined the profile of COX (6-keto prostaglandin $F_{1\alpha}$ (6-keto $PGF_{1\alpha}$), PGE_2, thromboxane B_2 (TXB_2) and NOS (nitrite and nitrate) metabolites released over a period of 10 days from segments of rat aorta. In each case, segments from the same animal were left untreated or treated with bacterial lipopolysaccharide (LPS; 10 $\mu g\ ml^{-1}$) in order to induce iNOS and COX-2. Prostaglandins were measured by radioimmunoassay whilst nitrite and nitrate were measured, respectively, by Greiss reaction alone, or following a nitrate reductase step. The isoforms of NOS and COX responsible for metabolite release were characterized pharmacologically by use of inhibitors and at the molecular level by reverse transcription polymerase chain reaction with specific primers for iNOS, eNOS, COX-1 and COX-2. In separate experiments the role of the endothelium in the release of nitrite, nitrate and prostaglandins and in the expression of iNOS, eNOS, COX-1 and COX-2 was determined by comparing responses in endothelium denuded and endothelium-intact segments of rat aorta

3 Under control culture conditions vessels released prostaglandins in the following rank order 6-keto $PGF_{1\alpha} = PGE_2 > > TXB_2$. LPS increased the release of 6-keto $PGF_{1\alpha}$ and PGE_2 but not of TXB_2, an effect that was inhibited by the protein synthesis inhibitor cycloheximide (1 μM), the anti-inflammatory steroid dexamethason (1 μM), the nonsteroidal anti-inflammatory drug indomethacin (30 μM) and, where tested, the selective COX-2 inhibitor NS-398 (30 μM). Similarly, segments of rat aorta released detectable levels of nitrite and nitrate, which were reduced by N^G-nitro-L-arginine methyl ester (L-NAME, 1 mM), which inhibits all isoforms of NOS, and by dexamethasone (1 μM), which inhibits the induction of iNOS. The proportion of nitrate to nitrite released over the 10 day period varied greatly from approximately 1:1 on days 5 to 8 to 5:1 on day 9. However, the sum of nitrite and nitrate (NOx) as well as PGE_2 remained elevated over the whole 10 day period. The formation of 6-keto $PGF_{1\alpha}$ peaked on days 1 and 2.

4 In freshly prepared tissue, mRNAs for eNOS, COX-1, iNOS and COX-2 were detected. After 24 h in culture, there was an apparent increase in the level of mRNAs for iNOS and COX-2 but not for eNOS or COX-1, an effect that was further enhanced when LPS was included in the culture medium. The expressions of mRNA for eNOS, COX-1, iNOS or COX-2 were not greatly different in vessels with intact or disrupted endothelium. Similarly the release of NOx or PGE_2 by vessels after the 1st or 9th day in culture were not significantly different from vessels prepared with or without endothelium.

5 Thus, COX-2 and iNOS are co-induced in intact vessels in culture, with the vascular smooth muscle being the main site of mediator generation. In contrast to data from isolated cells in culture (observed usually over 1 day), both COX and NOS activities in cultured blood vessels were elevated for at least 10 days. Also, unlike isolated cells in culture, the COX and NOS pathways were active independently; L-NAME had little effect on the activity of COX and indomethacin had little effect on the activity of NOS.

Keywords: Inducible cyclo-oxygenase; prostacyclin; prostaglandin E_2; thromboxane B_2; inducible nitric oxide synthase; nitric oxide

Introduction

The release of mediators, such as prostacyclin (PGI_2; Moncada *et al*., 1976) and nitric oxide (NO; Palmer *et al*., 1987) by blood vessels, represents an important part of vascular homeostasis. NO (Murad *et al*., 1978; Rees *et al*., 1989) and PGI_2 (Moncada *et al*., 1976) are vasodilators with potent inhibitory actions on platelet function (Moncada *et al*., 1976; Radomski *et al*., 1987; Sneddon & Vane, 1988). In addition, PGI_2 is considered to be an important endogenous anti-lipidaemic agent (see Willis *et al*., 1986).

In the healthy blood vessel, PGI_2 (Moncada *et al*., 1976) and NO (Furchgott & Zawadzki, 1980) are formed pre-

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dominately in the endothelium by constitutive isoforms of NO synthase (NOS; Pollock *et al*., 1991; Mitchell *et al*., 1991) and cyclo-oxygenase (COX-1; Mitchell *et al*., 1993), respectively. However, under some conditions, such as when exposed to bacterial lipopolysaccharide (LPS), vascular smooth muscle cells can also release NO (Busse & Mulsch, 1990; Thorin-Trescases *et al*., 1995) and PGI_2 (Bailey *et al*., 1985), a phenomenon associated with the expression of distinct isoforms of NOS (iNOS; see Moncada *et al*., 1991) and COX (COX-2; see Mitchell *et al*., 1995a). The induction of iNOS (Beasley, 1990; Jolou-Schaeffer *et al*., 1990; Wright *et al*., 1992; Boughton-Smith *et al*., 1993; Mitchell *et al*., 1992) or COX-2 (Vane *et al*., 1994; Masferrer *et al*., 1994; Tomlinson *et al*., 1994) *in vivo* is generally associated with deleterious responses such as hypotension, plasma extravasation and pain. Alternatively, there may be situations in which the induction of iNOS and/or COX-2 results in the release of anti-inflammatory mediators. Indeed, the local induction of iNOS (Hansson *et al*., 1994) or COX-2 in damaged vessels (Rimarchin *et al*., 1994), may represent a defence mechanism (Zembowicz *et al*., 1995) to compensate for endothelial dysfunction.

Studies demonstrating that iNOS and COX-2 are co-induced *in vitro* have mainly employed macrophages or macrophage-like cells (Stadler *et al*., 1991; 1993; Inoue *et al*., 1993; Salvemini *et al*., 1993; Swierkosz *et al*., 1995a,b). However, a co-induction has been implied from experiments in rat isolated aortic vascular smooth muscle cells in culture (Inoue *et al*., 1993), although expression of COX-2 was not confirmed in this study. The co-induction of iNOS and COX-2 is associated with 'cross-talk' between the two pathways with NO and prostanoids having both inhibitory and stimulating effects on COX-2 (Stadler *et al*., 1991; 1993; Salvemini *et al*., 1993; Swierkosz *et al*., 1995a,b) and iNOS (Marrota *et al*., 1992; Gaillard *et al*., 1992), respectively. These discrepancies in the literature may well be related to the considerable differences that can exist between cells held in culture in different laboratories. We were interested to study the temporal relationships and possible interactions between COX and NOS activities in blood vessels. However, due to the limitations of using isolated cells in culture we have utilized an organ culture system in order to answer these questions. Indeed, this approach is considered superior to the use of monolayers of isolated cells to study the responses of vascular tissue to injurious or atherogenic stimuli (Pederson & Bowyer, 1986; Koo & Gotlieb, 1989; Soyombo *et al*., 1990; 1995; Holt *et al*., 1994). Thus, using rat aorta in culture we have determined the pattern and ratio of release of COX and NOS metabolites over a 10 day time course. In addition, we have investigated the role of the endothelium in these responses and assessed any 'cross-talk' between the two enzymatic pathways. Some of these findings have been published in abstract form (Bishop-Bailey *et al*., 1996).

Methods

Preparation of rat aorta

Male Sprague-Dawley rats (250–300 g) were killed by overdose of sodium pentobarbitone (350 mg kg^{-1}; i.p.), and aortae (thoracic and abdominal) were immediately removed and placed into sterile pots containing phosphate buffered saline (PBS) supplemented with penicillin (1000 u ml^{-1}) and streptomycin (1 mg ml^{-1}), PBS; Pen-Strep, and stored at 5°C for less than 2 h before preparation.

Vessel organ culture

Under sterile tissue culture conditions, vessels were dissected free of connective tissue and washed 4–6 times in PBS; Pen-Strep to remove any adhered blood clots. Vessels were then cut into rings of approximately 2–3 mm width. Individual rings were then placed into wells of sterile 48-well plates containing 500 μl Dulbecco's modified Eagle medium (DMEM) containing 1 mM sodium pyruvate and phenol red, supplemented with Pen-Strep, 2 mM glutamine and 10% foetal calf serum (FCS), unless stated otherwise. All tissue incubations were carried out at 37°C, in an atmosphere of 5% CO_2, with a culture incubator (IR 1500 automatic CO_2 incubator; Flow Laboratories). Vessels were then left to equilibrate for 1 h before the medium was replaced with fresh medium containing drugs or vehicle.

From preliminary studies conducted over 48 h, the optimum concentration of LPS (*E. Coli*; serotype 0111 : B4) to induce the release of 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) was found to be 10 μg ml^{-1} (data not shown). This concentration of LPS was therefore used throughout the study. Vessels were incubated with LPS (and drug or vehicle) for up to 10 days with continual replacement of medium and drugs every 24 h. At these time points the medium was removed and accumulated nitrite measured immediately, samples were then frozen (−20°C) for later determination of nitrate and/or prostanoids levels. From day 5 onwards there was evidence of cells explanting on to the base of the 48 well plate. By day 10 these were greater than 90% confluent (data not shown).

Measurement of NO release

In aqueous solutions NO has a very short half-life and is oxidized to nitrite and nitrate (see, Butler *et al*., 1995). NO production was determined by measuring the accumulation of these breakdown products in the culture medium by a two step-procedure. Nitrite was measured immediately by the Greiss reaction. Nitrate was measured in the same samples by the Greiss reaction following reduction back to nitrite. Total NO production was then taken to be the sum of nitrite and nitrate, denoted by NOx.

Greiss reaction

Nitrite was measured spectrophotometrically by the Greiss reaction adapted for the 96-well plate reader (Schmidt *et al*., 1992). Briefly, 100 μl of sample medium was incubated with 100 μl of Greiss reagent (sulphanilamide [0.5%], ortho-phosphoric acid [2.5%] and N-(1-naphthyl)ethyl-enediamine [0.05%]) for 5 min in 96-well plates. The OD_{550}, was measured by a Titertek Multiskan MCC/340 MK. II plate reader and nitrite concentration calculated with solutions of sodium nitrite in DMEM (0–100 nmol ml^{-1}). Under these conditions the detection limit of the Greiss reaction was 1 nmol ml^{-1}.

Reduction of nitrate to nitrite

Nitrate concentration was assessed following stoichiometric reduction of nitrate to nitrite. Samples were incubated for 5 min at 37°C, before the addition of 0.1 unit ml^{-1} nitrite reductase (*Aspergillus* species), 50 μM NADPH and 5 μM FAD, and the reaction continued for a further 15 min at 37°C. NADPH, which absorbs at OD_{550} was oxidized by the addition of 10 unit ml^{-1} lactate dehydrogenase (rabbit muscle) and 10 mM sodium pyruvate for a further 5 min at 37°C (final volume 100 μl). Under these conditions, nitrate concentrations of up to 10 nmol ml^{-1} were completely converted to nitrite. The newly reduced nitrite was determined as above by the Greiss reaction. The culture medium used in this study contained 5–20 nmol ml^{-1} nitrate, therefore all samples were diluted (at least 1 : 5) in h.p.l.c. grade water before NOx could be determined. Tissue derived NOx was determined after subtraction of NOx present in batch matched DMEM, supplemented with FCS.

Tissue-induced acidification of culture medium

In preliminary experiments, vessel segments of approximately 10 mg (8.1 ± 0.6 mg) caused acidification of the culture medium as indicated by a colour change in the phenol red from red to yellow. The degree of acidification (measured by a change in

optical density at 550 nm) correlated with the concentration of NOx released by the vessel. Thus, for further experiments smaller tissue pieces of 3.3 ± 0.2 mg were used, and no acidification of the medium was observed.

Prostanoid determination

6-Keto $PGE_{1\alpha}$ (the breakdown product of PGI_2), thromboxane (TX) B_2 (the breakdown product of TXA_2), and PGE_2 were measured by radioimmunoassay by use of commercial antibodies and tritiated prostanoids, as previously described (Mitchell *et al*., 1993). Antibodies to 6-keto $PGF_{1\alpha}$ or TXB_2 did not cross-react with disparate prostanoids. However, PGE_2 cross-reacted approximately 15% with the antibody to 6-keto $PGF_{1\alpha}$ (Akarasereenont *et al*., 1995).

Measurement of COX-1, eNOS, COX-2, and iNOS expression in rat aorta by reverse transcription polymerase chain reaction

Rat aorta were prepared as above and segments either placed into culture for 24 h with or without LPS before being frozen, or frozen immediately (freshly elicited sample). Tissues were then stored at $-80°C$ until RNA was extracted. Total RNA was then extracted from the tissues by homogenization and converted to cDNA by standard methods. Polymerase chain reaction was performed with an OmniGene thermocycler. Initial denaturation was done at 94°C for 2 min followed by 28–36 cycles of amplification. Each cycle consisted of 35 s of denaturation at 94°C, 35 s of annealing at 58°C, and 45 s for enzymatic primer extension at 72°C. After the final cycle, the temperature was held at 72°C for 10 min to allow reannealing of amplified products. Polymerase chain reaction products were then size fractionated through a 2% agarose gel and the bands visualized with ethidium bromide. The sequences for primers were selected according to the published sequences in GenBank and were as follows: for rat COX-1 (160 base pairs) sense, 5′-TAA GTA CCA GGT GCT GGA TGG-3′; antisense, 5′-AGA TCG TCG AGA AGA GCA TCA-3′; for rat COX-2 (242 base pairs) sense, 5′-TCC AAT CGC TGT ACA AGC AG-3′; antisense, 5′- TCC CCA AAG ATA GCA TCT GG-3′; for rat eNOS (164 base pairs) sense, 5′-CGA GAT ATC TTC AGT CCC AAG C-3′; antisense, 5′-GTG GAT TTG CTG CTC THT AGG-3′; rat iNOS (305 base pairs) sense, 5′-TCT GTG CCT TTG CTC ATG AC-3′; antisense, 5′-CAT GGT GAA CAC GTT CTT GG-3′. Rat glyceraldehyde-3-phosphate dehydrogenase (G3PDH), a constitutively expressed gene, was chosen as a control. The sequence for G3PDH (452 base pairs) was as follows, sense 5′-ACC ACA GTC CAT GCC ATC AC-3′ and for antisense 5′-TCC ACC ACC CTG TTG CTG TA-3′.

Materials

Nitrate reductase and lactate dehydrogenase were from Boehringer-Mannheim U.K., (Lewes, East Sussex); tritiated prostanoids were from Amersham (Slough, Berks); NS-398 (N-(2-cyclohexyloxy-4-nitrophenyl) methanesulphonamide) was purchased from Calbiochem (Nottingham); Dulbecco's modified Eagle medium (DMEM), foetal calf serum, penicillin, streptomycin and glutamine were supplied by Gibco BRL (Paisley, Renfewshire, Scotland); lipopolysaccharide (*E. Coli*; serotype 0111 : B4), polyclonal antibodies to 6-keto $PGF_{1\alpha}$, PGE_2 and TXB_2 salicylic acid (sodium salt), acetylsalicylic acid, indomethacin, sodium nitrite, sodium nitrate, N^G-nitro-L-arginine methyl ester, N^G-nitro-D-arginine methyl ester, cycloheximide, dexamethasone, hydrochloric acid, Greiss reagent (sulphanilamide (0.5%), ortho-phosphoric acid (2.5%) and N-[1-naphthyl]ethyl-enediamine (0.05%)) were from Sigma Chemical Co. (Poole, Dorset). LPS was dissolved and diluted in sterile water. NS-398 was dissolved in cell culture grade DMSO, and diluted in DMEM. Salicylic acid (sodium form), acetylsalicylic acid and indomethacin were dissolved in sodium carbonate (5%), all other drugs were made up in sterile distilled water. These solutions were then filtered through a 0.2 μm filter, and all subsequent dilutions made up in DMEM.

Statistics

Statistical analyses were by one sample and unpaired *t* tests, performed by Instat 2.04 (Graphpad Software). A P value of <0.05 was taken as significant.

Results

Time-dependent release of nitrite and nitrate by segments of rat aorta

Segments of rat aorta maintained in culture for up to 10 days released detectable amounts of NOx throughout the time course, with the highest release being detected on days 2 and 9 (Figure 1). In the presence of LPS (10 μg ml^{-1}) rat aortic segments released increased amounts of NOx (Figure 1). During the peaks of NOx generation (days 2 and 9), nitrate was released in 3 and 5 fold greater amounts than nitrite, respectively. Throughout the remainder of the time course nitrite and nitrate were released in approximately equal amounts (Table 1). In separate experiments the release of NOx (nmol ml^{-1} mg^{-1}) during day 1 (basal release 2.2 ± 1.1; after LPS, 6.3 ± 1.5) was not altered when the endothelium was intentionally removed by mechanical rubbing (basal release, 1.1 ± 0.7; after LPS, 5.5 ± 1.1) ($n = 3$). Similarly on day 9, NOx release (basal release, 5.3 ± 1.4; after LPS, 16.7 ± 1.6) was slightly, although not significantly (two-way ANOVA) reduced in endothelium-denuded rings (basal release, 2.5 ± 1.4; after LPS, 13.6 ± 0.9) ($n = 4$).

Pharmacological characterization of the isoform of NOS responsible for NOx release by rat aorta in culture

The elevated production of NOx induced by LPS on day 2 was reduced to control (vehicle treated) levels by co-incubation with N^G-nitro-L-arginine methyl ester (L-NAME; 1 mM; Figures 1 and 2), or dexamethasone (1 μM; Figure 2), but was unaffected by N^G-nitro-D-arginine methyl ester (D-NAME 1 mM; Figure 2). Co-incubation of aorta with LPS plus

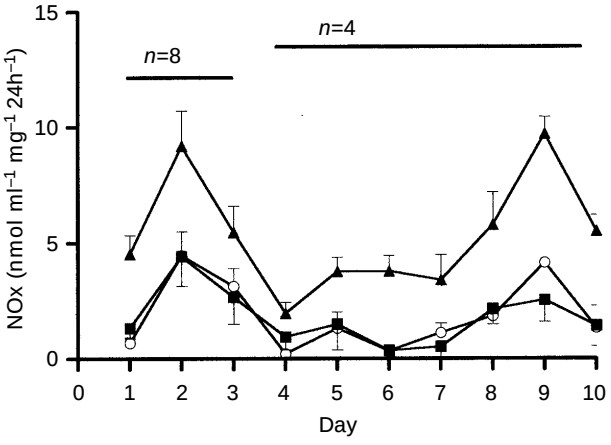

Figure 1 Time course of NOx release from rat aortic segments under control culture conditions (■), in the presence of LPS (10 μg ml^{-1}, ▲), or in the presence of LPS and L-NAME (1 mM; ○). The medium, vehicle, LPS and/or L-NAME were replaced every 24 h for 10 days. The results represent the mean from $n = 4-8$ animals; vertical lines show s.e.mean. Each *n* number was determined as the mean of incubations made in triplicate.

Table 1 The ratio of nitrate (NO_3^-) to nitrite (NO_2^-) released from segments of rat aorta in control culture conditions (control) or in the presence of LPS (10 μg ml^{-1}, every 24 h) over 10 consecutive days

Day	NO_2^- Control	LPS	NO_3^- Control	LPS	Ratio LPS NO_3^-:NO_2^-
1	0.7±0.2	1.8±0.2*	0.9±0.6	4.2±0.9*	3.0±0.7
2	2.0±0.8	4.0±0.2*	4.9±1.0	8.9±1.0*	2.7±0.5
3	1.5±0.5	3.1±0.1*	3.2±1.3	5.3±0.6	1.7±0.1
4	1.3±0.4	1.7±0.2	0.0±0.8	0.4±0.5	0.8±0.3
5	0.4±0.3	2.1±0.2*	1.1±0.8	1.7±0.5	1.5±0.4
6	0.1±0.1	2.3±0.3*	0.3±0.3	1.5±0.4*	0.8±0.1
7	0.1±0.1	1.8±0.2*	0.4±0.4	1.6±1.0	1.5±0.4
8	0.3±0.1	3.8±0.3*	1.8±0.6	2.0±1.3	0.8±0.2
9	0.4±0.1	1.6±0.1*	2.2±0.8	8.1±0.6*	5.2±0.3
10	0.1±0.0	2.5±0.2*	1.3±0.8	3.0±0.6	1.2±0.2

The data were calculated by dividing the amount of nitrate by that of nitrite in each sample from individual animals. These data represent the mean±s.e.mean from 12 (days 4–10) or 24 (days 1–3) incubations from 4–8 animals. *Denotes significant ($P<0.05$; unpaired t test) difference in nitrite or nitrate release in the presence of LPS compared to control culture conditions.

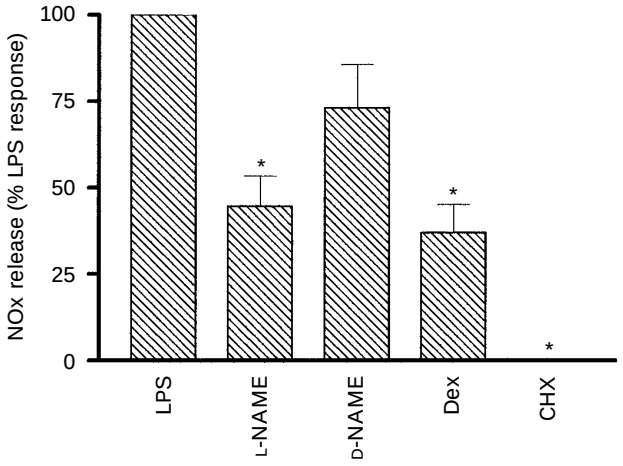

Figure 2 Characterization of the increase in NOx production induced by LPS (10 μg ml^{-1}) by segments of rat aorta on day 2. L-NAME (1 mM), dexamethasone (Dex, 1 μM) and cycloheximide (CHX; 1 μM) but not D-NAME (1 mM), inhibited NOx release. The data represents the mean±s.e.mean of $n=8$ animals. Each n number was derived from the mean of triplicate incubations. *Denotes significant difference ($P<0.05$; one sample t test) in NOx release compared to LPS alone. Similar results were obtained when mediator release was measured on days 1 and 3.

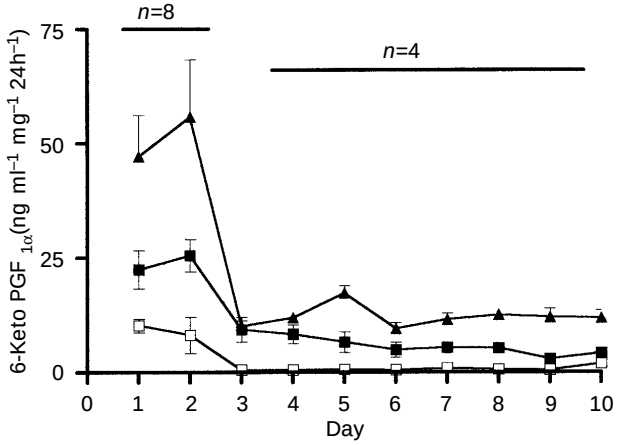

Figure 3 Time course of 6-keto $PGF_{1\alpha}$ release from rat aortic segments in organ culture under control culture conditions (■), in the presence of LPS (10 μg ml^{-1}; ▲) or LPS plus indomethacin (30 μM, □). Results represent the mean from $n=4-8$ animals; vertical lines show s.e.mean. Each n number was determined by the means of triplicate incubations.

cycloheximide (CHX 1 μM) virtually abolished NOx release (Figure 2). In separate experiments L-NAME (1 mM) significantly ($P<0.05$; paired t test) inhibited the release of NO (nmol ml^{-1} mg^{-1}) from segments of rat aorta (day 2; $n=11$) maintained under control culture conditions (control 2.7±0.7; plus L-NAME 0.8±0.5).

Time-dependent release of prostanoids from segments of rat aorta

Segments of rat aorta maintained in culture for up to 10 days released 6-keto $PGF_{1\alpha}$ (Figure 3), PGE_2 (Figure 4) and TXB_2 (Figure 5). Two peaks of PGE_2 production were detected, at days 1–3 and days 6–9. This contrasted with a single peak of 6-keto $PGF_{1\alpha}$ at days 1–2. The release of both PGE_2 and 6-keto $PGF_{1\alpha}$ was enhanced by LPS (Figure 3 and 4; Table 2). Conversely, TXB_2 release was not potentiated by LPS at any time point (Figure 5). Segments of rat aorta under control culture conditions released low levels of 6-keto $PGF_{1\alpha}$ (ng ml^{-1} ng^{-1}) over 1 h (10±1) and 6 h (8±1). This acute release of 6-keto $PGF_{1\alpha}$ was not enhanced by the presence of LPS (1 h, 8±1; 6 h, 10±1). In separate experiments the release of PGE_2 (ng ml^{-1} mg^{-1}) during day 1 (basal release 44.3±2.6 after LPS, 64.8±3.1) was not altered when the endothelium was intentionally removed by mechanical rubbing (basal release, 35.8±2.8; after LPS, 68.1±4.2) ($n=3$).

Pharmacological characterization of the isoform of COX responsible for prostanoid release by rat aorta in culture

Indomethacin (30 μM) inhibited the elevated production of 6-keto $PGF_{1\alpha}$ induced by LPS throughout the time course (Figure 3). Similarly, where tested (days 1–3), dexamethasone (1 μM) and cycloheximide (1 μM) significantly inhibited LPS-induced production of 6-keto $PGF_{1\alpha}$ (Figure 6a). In separate experiments (day 1) NS-398 (inhibited by 79±10%), indomethacin (inhibited by 63±6.5%) and aspirin (inhibited by 36±19.5%) but not sodium salicylate (inhibited by 26±13%; not significant), at a common concentration (30 μM) inhibited 6-keto $PGF_{1\alpha}$ released by segments of rat aorta under control culture conditions ($n=5-6$) and exposed to LPS (Figure 6b). NS-398 had the greatest effect of all NSAIDs (Figure 6b).

Characterization of potential 'cross-talk' between the COX and NOS pathways in rat aorta in culture

NS-398 (30 μM), indomethacin (30 μM), aspirin (30 μM) or sodium salicylate (30 μM) had no effect on LPS induced NOx generation measured up to 3 days (data not shown). L-NAME (1 mM) modestly inhibited LPS induced 6-keto $PGF_{1\alpha}$ release on day 1 (by 32±10%; $n=16$; $P<0.05$; one sample t test), but

Table 2 The ratio of PGE_2 to 6-keto $PGE_{1\alpha}$ released from segments of rat aorta cultured without (control) or with LPS (10 μg ml^{-1}) over 10 consecutive days

	6-Keto $PGE_{1\alpha}$		PGE_2		Control	LPS
Day	*Control*	*LPS*	*Control*	*LPS*	*PGE_2:6-keto*	*PGE_2:6-keto*
1	22.5±4.3	47.1±9.1*	23.9±7.3	55.6±11.1*	1.0±0.2	1.2±0.3
2	25.5±3.5	55.8±12.5*	50.0±9.3	60.5±5.8	2.1±0.3	2.6±0.2
3	9.3±2.8	10.1±1.1	20.6±6.8	14.5±2.1	2.3±0.4	1.9±0.2
4	8.3±2.1	11.9±1.0	27.3±6.6	35.6±3.8	3.2±0.3	2.8±0.3
5	6.6±2.3	17.3±1.6*	24.5±10.0	39.0±11.0	3.3±0.4	2.7±0.7
6	4.9±1.6	9.5±1.3	18.8±5.7	32.9±5.2	3.7±0.3	3.2±0.4
7	5.4±1.1	11.5±1.3*	15.8±4.6	54.7±5.1*	2.8±0.6	5.0±0.4
8	5.2±1.1	12.5±0.6*	28.1±5.7	47.3±3.0*	5.6±1.0	3.5±0.6
9	2.9±0.3	12.0±1.9*	10.4±1.4	42.5±5.0*	3.7±0.5	4.1±0.7
10	4.2±0.3	11.8±1.7*	8.8±1.3	25.5±3.9*	2.1±0.3	3.2±0.3

The data were calculated by dividing the amount of PGE_2 by 6-keto $PGF_{1\alpha}$ in each individual incubation. These data represent the mean±s.e.mean from 12 (days 4–10) or 24 (days 1–3) incubations from 4–8 animals. *Denotes significant ($P<0.05$; unpaired t test) difference in prostanoid release in the presence of LPS compared to control values.

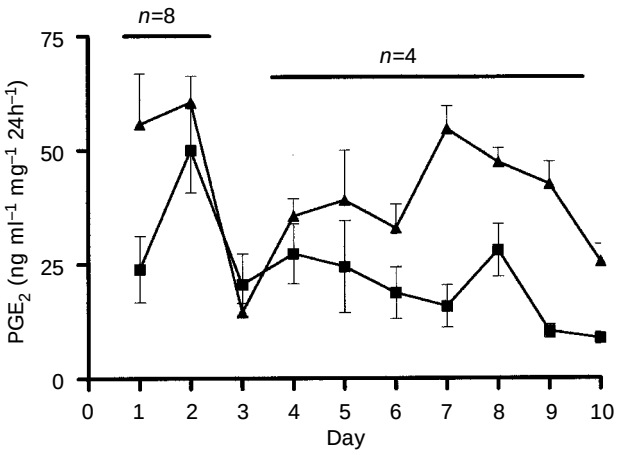

Figure 4 Time course of PGE_2 release from rat aortic segments in organ culture under control culture conditions (■) or in the presence of LPS (10 μg ml^{-1}; ▲). Results represent the mean from $n=4-8$ animals; vertical lines show s.e.mean. Each n number was determined by the means of triplicate incubations.

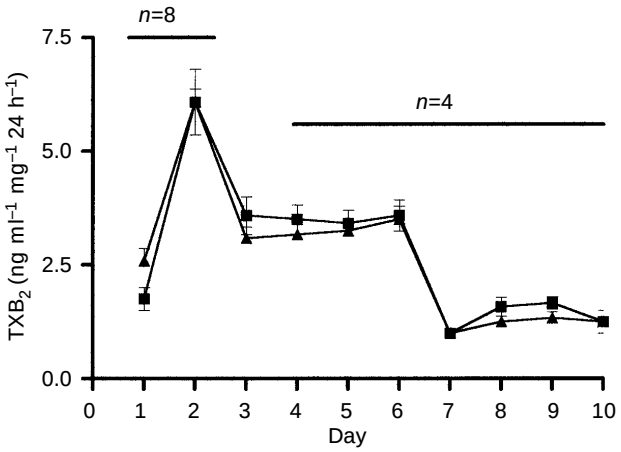

Figure 5 Time course of TXB_2 release from rat aortic segments in organ culture under control culture conditions (■) or in the presence of LPS (10 μg ml^{-1}, ▲). Results represent the mean from $n=4-8$ animals; vertical lines show s.e.mean. Each n number was determined by the mean of triplicate incubations.

not on day 2 (increased by 4±15%; $n=8$) or on subsequent days (data not shown). Moreover, when all time points were considered there was no significant difference calculated by two-way ANOVA between the release of 6-keto $PGF_{1\alpha}$ in the presence or absence of L-NAME. Similarly L-NAME had no significant effect on the release of 6-keto $PGF_{1\alpha}$ from tissues under control culture conditions on day 1 (reduced by 20±11%) or day 2 (reduced by 17±21%), $n=4$.

Molecular characterization of the isoforms of NOS and COX present in rat aorta after organ culture

In freshly prepared tissue, mRNA for the constitutive enzymes COX-1 (Figure 7a) and eNOS (Figure 7c) and for the inducible enzymes iNOS (Figure 7b) and COX-2 (Figure 7d) were detected. After 24 h in organ culture mRNAs for iNOS and COX-2, but not for eNOS or COX-1 were apparently increased. There appeared to be a further increase in the expression of COX-2 and iNOS when LPS was included in the culture medium, whereas eNOS and COX-1 levels were unchanged. The levels of mRNA for iNOS, COX-2, eNOS or COX-1 in vessel segments were not greatly different in tissues prepared with or without endothelium (Figure 7).

Discussion

Using an organ culture system, we have shown that intact vessel segments can continue to express the inducible enzymes, iNOS and COX-2, for much longer time periods than was previously appreciated. Moreover, we have shown that the vascular smooth muscle layer is the main site of expression of these enzymes.

Using reverse transcription polymerase chain reaction, we found that freshly elicited tissue expressed mRNA for both constitutive and inducible forms of NOS and COX. The constitutive expression of iNOS and COX-2 was not expected as these isoforms are thought only to be expressed after inflammatory insults. However, other studies have described 'constitutive' expression of iNOS in rat (Akyurek *et al*., 1996) and human (Park *et al*., 1996) tissue and of COX-2 in human tissue (O'Neill & Ford-Hutchinson, 1993). Nevertheless, after 24 h in culture, tissues appeared to express increased levels of mRNA for the inducible enzymes iNOS and COX-2, whilst the levels of eNOS and COX-1 remained unchanged. Moreover, mRNAs for iNOS and COX-2, but not eNOS or COX-1, were further increased when LPS was included in the culture medium, an observation that is consistent with previous studies in isolated cells in culture (see Mitchell *et al*., 1995b). In parallel with the increase in expression of iNOS and COX-2 mRNA, vessel segments released increased amounts of NOx and prostanoids after exposure to LPS. The induced release of NOx and prostanoids was inhibited by the protein synthesis inhibitor, cycloheximide suggesting that the newly synthesized iNOS and COX-2 were responsible for the observed mediator release. Similarly, dexamethasone inhibited the release of both NOx and prostanoids induced by LPS. Dexamethasone has been extensively shown to inhibit the expression of iNOS en-

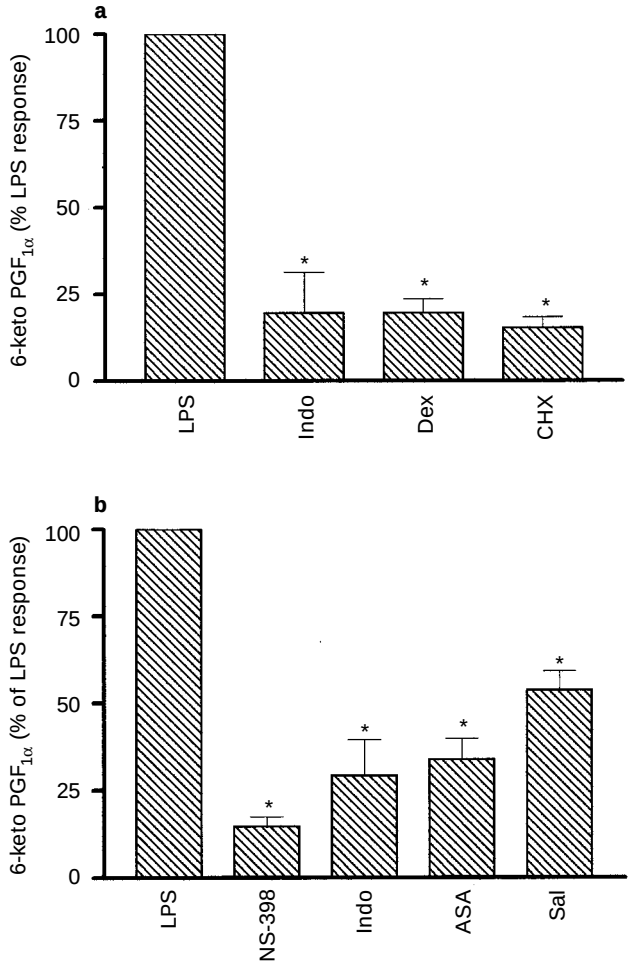

Figure 6 Characterization of the LPS (10 μg ml^{-1})-induced release of 6-keto $PGF_{1\alpha}$ by segments of rat aorta on day 2. Indomethacin (Indo; 30 μM), dexamethasone (Dex; 1 μM) and cycloheximide (CHX; 1 μM) significantly inhibited the release of 6-keto $PGF_{1\alpha}$ induced by LPS. The data represent the mean $\pm$ s.e.mean of tissues from $n=8$ animals, each n number being the mean of duplicate or triplicate incubations. *Denotes significant ($P<0.05$; one sample t test) difference in 6-keto $PGF_{1\alpha}$ release compared to LPS alone. The results shown were obtained on day 2. Similar results were obtained when mediator release was measured on days 1 and 3. (b) Comparison of the effect of NS-398, indomethacin, aspirin (ASA) and sodium salicylate (Sal) on the release of 6-keto $PGF_{1\alpha}$ by segments of rat aorta cultured in the presence of LPS (10 μg ml^{-1}). The data represent the mean $\pm$ s.e.mean for $n=4-13$ animals, each n number being the mean of single or duplicate incubations. *Denotes significant ($P<0.05$; one sample t test) difference in the release of 6-keto $PGF_{1\alpha}$. The results obtained on day 1 are shown. Similar results were obtained on days 2 and 3.

zyme without effecting the expression of constitutive NOS isoforms (Radomski *et al*., 1990), supporting our conclusion that the NOx detected is formed by iNOS. Dexamethasone also reduced the production of prostaglandins. This could be due either to it inhibiting the induction of COX-2 (Mitchell *et al*., 1994) protein, or to it inhibiting the release of substrate (see Mitchell *et al*., 1995a). Sensitivity to dexamethasone cannot therefore be used to discriminate between COX-1 and COX-2, as it can discriminate between iNOS and eNOS. Traditional NSAIDs inhibit both COX-1 and COX-2 and so are not useful as tools to discriminate the activities of COX-1 from COX-2. However, NS-398 is a selective inhibitor of COX-2 and can therefore be used in comparison with traditional non-steroidal anti-inflammatory drugs (NSAIDs) in order to elucidate the roles of COX-1 or COX-2 in the release of prostaglandins under different conditions. Using a common concentration of 30 μM (at which NS-398 is a COX-2 specific; Futaki *et al*., 1994), we found NS-398 produced a greater degree of prostanoid inhibition than indomethacin, aspirin or sodium salicylate. This order of efficiency is in agreement with the described potency of these drugs on COX-2 isoforms (Mitchell *et al*., 1993; Meade *et al*., 1993; Futaki *et al*., 1994). Sodium salicylate inhibited prostanoid release more effectively in LPS treated vessels than in untreated tissue. This observation may reflect the greater contribution of COX-2 to the release by vessels treated with LPS, where sodium salicylate may inhibit transcription of cDNA encoding COX (Wu *et al*., 1991) or arachidonic acid availability (Mitchell *et al*., 1995b).

Taken together, therefore, our molecular biological and pharmacological evidence suggest that during inflammatory events in blood vessels the inducible forms of NOS and COX are responsible for the majority of mediator release. As the release of NOx and PGE_2 as well as the expression of iNOS and COX-2 were similar in vessel segments prepared with or without endothelium our data also suggest that the vascular smooth muscle is the major site for iNOS and COX-2 expression and metabolite formation under these conditions.

The ratio of the amounts of prostanoids produced by rat aorta changed over the 10 day period. For instance, approximately equal amounts of 6-keto $PGF_{1\alpha}$ and PGE_2 were formed on days 1 and 2 whilst PGE_2 was produced in greater amounts on subsequent days. These findings are in agreement with others which have indicated that there is a large pool of constitutively expressed PGI_2 synthetase in healthy rabbit (Eldor *et al*., 1981), bovine (DeWitt & Smith, 1983) or human (Kerstein *et al*., 1983) vascular tissue. A similar pattern of PGI_2 and PGE_2 release was osberved with porcine aortic smooth muscle cells in culture (Ager *et al*., 1982), where a down regulation of PGI_2 synthetase, possibly due to inhibition by hydroperoxyfatty acids (Salmon *et al*., 1987) was demonstrated (Ager *et al*., 1982). Alternatively, tissue sulphydryl levels may change during the 10 day culture period favouring the production of PGE_2 (Ager *et al*., 1982). The small release of TXB_2 detected from the vessels in culture is consistent with the vessel containing low amounts of TXA_2 synthetase (Kerstein *et al*., 1983).

The release of NOx by segments of rat aorta could be divided into two components, one sensitive to L-NAME and dexamethasone, and a residual amount which was inhibited by cycloheximide. This residual release of NOx is therefore unlikely to be solely derived from NOS and may be due to protein catabolism, or NO derived from cytochrome P450 pathways (Boucher *et al*., 1992) or derived from tissue nitrite (Zweier *et al*., 1995).

In biological systems conversion of NO in aqueous solution to nitrite and nitrate is thought to favour nitrite production (see, Butler *et al*., 1995). Indeed, in murine macrophages treated with LPS this ratio is a constant 3 : 2, nitrite : nitrate (Steuhr & Marletta, 1987). However, the ratio of nitrite to nitrate released by rat aorta changed throughout the time of incubation (Table 1), with nitrate being the predominant product on days 2 and 9. This altered ratio of nitrite to nitrate may represent differences in the tissue levels of anti-oxidants such as superoxide dismutase. For example, manganese superoxide dismutase can be unregulated by cytokines (Wong & Goeddel, 1988). Interestingly, the breakdown of NO to higher oxides may be particularly prevalent in diseases such as atherosclerosis, in which large amounts of oxygen derived free radicals are produced. These findings show that it is essential to measure both nitrite and nitrate for an accurate determination of iNOS activity to be made.

Under some conditions (see Stadler *et al*., 1991; Marrota *et al*., 1992; Gaillard *et al*., 1992; Inoue *et al*., 1993; Salvemini *et al*., 1993; Swierkosz *et al*., 1995a,b; Mitchell *et al*., 1995a), but not others the co-induction of iNOS and COX-2 results in 'cross-talk' between the enzyme pathways. However, we found no effect of indomethacin, NS-398, aspirin or sodium salicylate on the production of NOx. This is contrary to the inhibition by NSAIDs of the induction of iNOS found in LPS and inter-

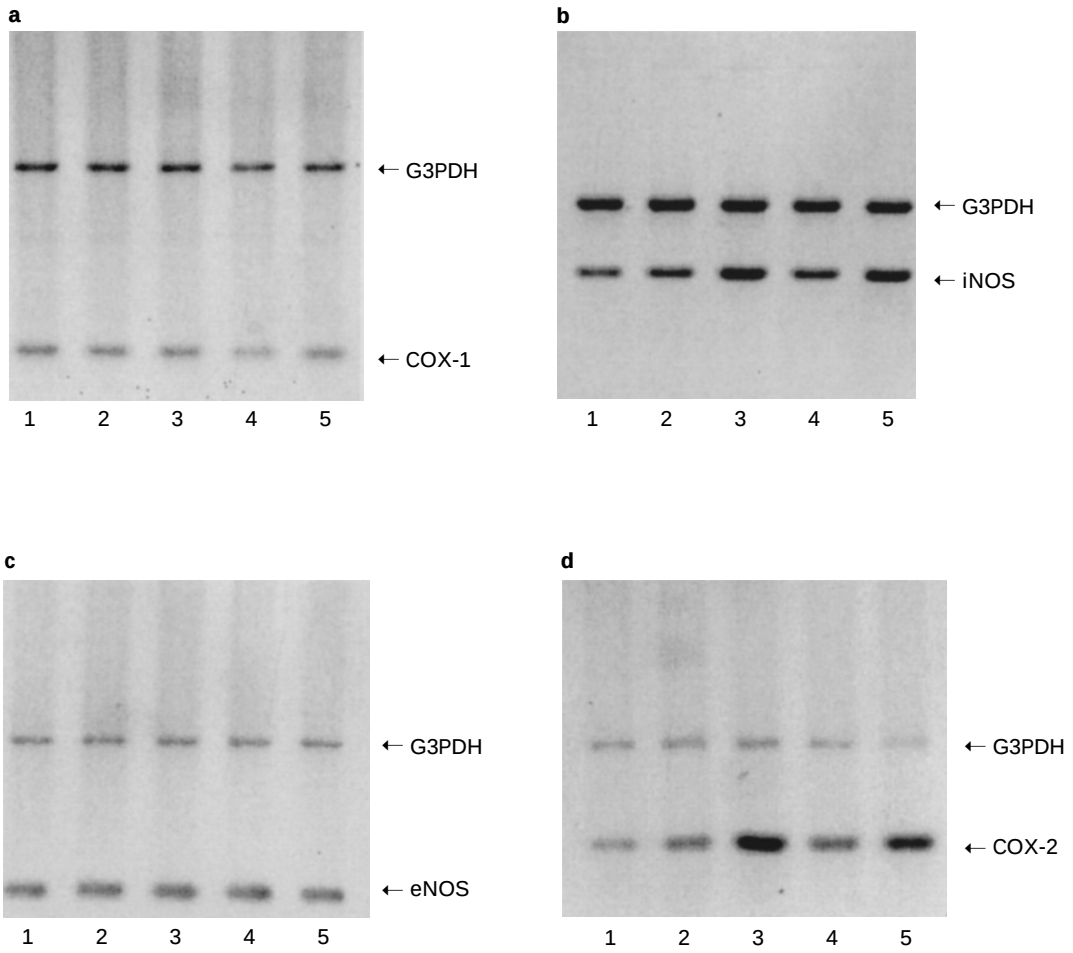

Figure 7 Expression of mRNA for COX-1 (a), iNOS (b), eNOS (c) and COX-2 (d) in segments of rat aorta prepared with or without endothelium. Lane 1, freshly elicited tissue (with endothelium intact); lane 2, tissue after organ culture for 24 h (with endothelium intact); lane 3, tissue after organ culture with LPS (10 μg ml^{-1}) for 24 h (with endothelium intact); lane 4, endothelium-denuded tissue after 24 h in organ culture; lane 5, endothelium-denuded tissue after 24 h in culture plus LPS. For each gel the respective mRNA (COX-1, 160 base pairs; eNOS, 164 base pairs; iNOS, 305 base pairs; COX-2, 242 base pairs) is indicated together with that for glyceraldehyde-3-phosphate dehydrogenase (G3PDH, 452 base pairs).

feron-γ treated rat alveolar macrophages (Aaederhard *et al*., 1995) or LPS treated mesangial cells (Gaillard *et al*., 1992), but in agreement with the lack of effect of these drugs on activated macrophages (Salvemini *et al*., 1993; Swierkosz *et al*., 1995a,b). Similarly, inhibition of the production of NO by L-NAME was without effect on 6-keto $PGF_{1\alpha}$ at most of the time points.

These findings show that COX and NOS activities can be elevated in intact vessels for prolonged periods. These observations may have relevance in diseases such as atherosclerosis, where inducing cytokines are continuously expressed in plaques. Thus, the study of how inflammatory enzymes are regulated in whole vessel culture may represent a useful model for human vascular disease.

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