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Comparison of the induction of cyclooxygenase and nitric oxide synthase by endotoxin in endothelial cells and macrophages

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Abstract

Endotoxin causes the expression of inducible nitric oxide (NO) synthase and cyclooxygenase-2. We have compared the ability of endotoxin to increase the activities of these enzymes in bovine aortic endothelial cells and the macrophage cell line (J774.2). Endotoxin (1 μg ml^{-1}; for 24 h) caused a time-dependent increase in the accumulation of cyclooxygenase metabolites from endogenous arachidonic acid, in both cell types. Cyclooxygenase activity towards exogenous arachidonic acid (30 μM; for 15 min) was also increased in both cell types. Endothelial cells and macrophages also contained comparable amounts of cyclooxygenase-2 protein after incubation with endotoxin for 24 h which was prevented by pretreatment with cycloheximide (10 μg ml^{-1}; 30 min prior to endotoxin). Endotoxin for 24 h caused a time-dependent increase in nitrite accumulation in macrophages, but not in endothelial cells. Thus, endotoxin increased cyclooxygenase activity and induced cyclooxygenase-2 protein in endothelial cells and macrophages. Endotoxin also increased NO synthase activity in macrophages, but not in endothelial cells.

Keywords: Cytokine; Lipopolysaccharide; Mitogen; Nitric oxide (NO); Prostaglandin

1. Introduction

The prostaglandins and nitric oxide (NO) are ubiquitous mediator systems which have numerous cardiovascular and inflammatory effects (Vane and Botting, 1992; Moncada et al., 1991). Cyclooxygenase is the first enzyme in the pathway in which arachidonic acid is converted to prostaglandins, prostacyclin and thromboxane A_2 (Vane and Botting, 1990; Smith and Marnett, 1991). This enzyme, like the one which forms NO from the guanidino group of L-arginine, NO synthase, exists in at least two isoforms. The constitutive isoforms, cyclooxygenase-1 and constitutive NO synthase, are present in many types of cells, while the inducible isoforms of cyclooxygenase (cyclooxygenase-2) and NO synthase are expressed after stimulation of cells with a variety of agents including endotoxin (bacterial lipopolysaccharide) and a number of cytokines and mitogens (Lee et al., 1992; Xie et al., 1992; see Thiemermann, 1994). The induction of cyclooxygenase-2 and NO synthase results in the increased synthesis of prostaglandins and NO. However, the mechanisms involved in this increased synthesis of prostaglandins and NO are complicated by the interaction between the products of the two systems (Marotta et al., 1992; Salvemini et al., 1993) and by the cytokines used for induction (Radomski et al., 1990; Schini et al., 1992; Pusztai et al., 1993). Here we have compared the degree of induction of cyclooxygenase-2 and inducible NO synthase caused by endotoxin in bovine aortic endothelial cells and macrophages, in order to explore potential differences between the responses of the two mediator systems. Some preliminary results of this work have been communicated to the British Pharmacological Society (Akarasereenont et al., 1994).

2. Materials and methods

2.1. Cell culture

Murine macrophages (J774.2; The European Collection of Animal Cell Culture; Salisbury, UK) were cul-

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tured in 96-well plates with Dulbecco's modified Eagle's medium (DMEM; 200 μl/well) containing 10% foetal calf serum (Gibco) and 4 mM L-glutamine. Bovine aortic endothelial cells were obtained from fresh bovine aortae as previously described (De Nucci et al., 1988) and cultured in 96-well plates as above. Each well was loaded with 10^5 endothelial cells or 1.3×10^6 macrophages. All agents were dissolved in distilled water and sterilised by filtration through a filter (pore size: 0.22 μm) before being added to the cells under sterile conditions. Cells were incubated at 37°C in a humidified incubator.

2.2. Measurement of the release of cyclooxygenase metabolites

Cyclooxygenase metabolites were measured by radioimmunoassay (Salmon, 1978) for 6-oxo-prostaglandin $F_{1\alpha}$, prostaglandin E_2, prostaglandin $F_{2\alpha}$ or thromboxane B_2. Antibodies to thromboxane B_2, prostaglandin E_2 and prostaglandin $F_{2\alpha}$ had less than 5% cross-reactivity with any of the other prostanoids measured. Antibody to 6-oxo-prostaglandin $F_{1\alpha}$ had less than 5% cross-reactivity to thromboxane B_2 and prostaglandin $F_{2\alpha}$, and 17% cross-reactivity to prostaglandin E_2. For experiments designed to measure the release of cyclooxygenase metabolites from endogenous arachidonic acid, cells were treated with endotoxin (1 μg ml^{-1}) for 3, 6, 12, or 24 h and the medium was subsequently removed for radioimmunoassay. In separate experiments designed to measure 'cyclooxygenase activity', cells were treated with endotoxin for 3, 6, 12, or 24 h after which time fresh medium containing arachidonic acid (30 μM) was added for 15 min at 37°C and then removed for radioimmunoassay. In some experiments, acetylsalicylic acid, indomethacin (both at 1, 10 or 100 μg ml^{-1}; 30 min prior to endotoxin), cycloheximide (10 μg ml^{-1}; 30 min prior to endotoxin) or dexamethasone (1 μM; 1 h prior to endotoxin) were added prior to a 24 h treatment with endotoxin and the predominant cyclooxygenase metabolite for bovine aortic endothelial cells (6-oxo-prostaglandin $F_{1\alpha}$) or macrophages (prostaglandin $F_{2\alpha}$) was measured as above.

2.3. Immunoblot (Western blot) analysis

Bovine aortic endothelial cells or macrophages with or without endotoxin (1 μg ml^{-1}) were cultured in T175 flasks (37°C; 24 h). Cells were washed with phosphate buffered saline (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; phenylmethylsulfonyl fluoride, 1 mM; pepstatin A, 0.05 mM 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; sodium dodecyl sulfate, 10% w/v; glycerol, 10% v/v; 2-mercaptoethanol 10% v/v and bromphenol blue, 2 mg ml^{-1}). Samples were centrifuged at $10000 \times g$ for 2 min before being loaded onto gradient gels (4–12% Tris-glycine; Novex) and subjected to electrophoresis (1.5 h at 125 V). The separated proteins were transferred to nitrocellulose (BioRad; 1 h at 200 V). After transfer to nitrocellulose, the blot was incubated in blocking solution (dried minimal-fat milk 5% w/v and Tween-20 0.25% v/v in phosphate buffered saline solution) for 1 h and then primed with primary antibody (dilution 1 : 1000), a rabbit antibody raised to murine cyclooxygenase-2 (Caymen Chemical Company, MI, USA; no detectable cross-reactivity with cyclooxygenase-1), for 1 h. The blot was then incubated with secondary antibody (dilution 1 : 1000), an anti-rabbit IgG developed in sheep, linked to alkaline phosphatase conjugate, for 1 h. Finally, the blot was developed for approximately 5 min with premixed solution (BCIP/NBT) containing 5-bromo-4-chloro-3-indolyl phosphate (BCIP), 0.56 mM; nitro blue tetrazolium (NBT), 0.48 mM; Tris, 10 mM; and $MgCl_2$, 59.3 mM; (pH 9.2). The detection limit of protein in cell extract was 1–10 ng of protein.

2.4. Measurement of NO synthase activity

NO synthase activity (3, 6, 12 and 24 h after endotoxin) was measured as the accumulation of nitrite in the culture medium using the Griess reaction adapted for a 96-well plate reader (Gross et al., 1991). Nitrite levels were measured in aliquots of the same culture medium used to determine cyclooxygenase metabolites. Nitrite was measured by adding 100 μl of Griess reagent (1% w/v sulphanilamide and 0.1% w/v naphthylethylenediamide in 5% v/v phosphoric acid) to 100 μl of sample culture medium. The optical density at 550 nm (OD_{550}) was measured using a Molecular Devices microplate reader (Richmond, CA, USA). Nitrite concentrations were calculated by comparison with OD_{550} of standard solutions of sodium nitrite prepared in culture medium. The detection limit for nitrite measurement in culture medium was 1 μM.

2.5. 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 and Levi, 1992). At the end of each experiment, cells in 96-well plates were incubated (37°C; 1 h) with MTT (0.2 mg ml^{-1}) dissolved in culture medium, after which time the medium was removed by aspiration and cells were solubilized in DMSO (200 μl). The extent of reduction of MTT to formazan within cells was quantitated by

measurement of optical density at 650 nm (OD_{650}) using a Molecular Devices microplate reader (Richmond, CA, USA).

2.6. Materials

Escherichia coli lipopolysaccharide (serotype: 0111:B4), aspirin, indomethacin, cycloheximide, dexamethasone, DMSO, phosphate buffered saline, Trizma base, EDTA, Triton X-100, phenylmethylsulfonyl fluoride, pepstatin A, leupeptin, glycerol, 2-mercaptoethanol, bromphenol blue, sulfanilamide, naphthylethylenediamide, phosphoric acid, sodium nitrite, sodium dodecyl sulfate, anti-rabbit IgG antibody, premixed BCIP/NBT solution, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 6-oxo-prostaglandin $F_{1\alpha}$, prostaglandin E_2, thromboxane B_2, prostaglandin $F_{2\alpha}$ and their respective antibodies were supplied by Sigma Chemical Company (Poole, UK). [^{3}H]6-oxo-Prostaglandin $F_{1\alpha}$, [^{3}H]prostaglandin E_2, [^{3}H]thromboxane B_2 and [^{3}H]prostaglandin $F_{2\alpha}$ were purchased from Amersham International (Buckinghamshire, UK). Dulbecco's modified Eagle's medium (DMEM) was obtained from Flow Laboratories. L-Glutamine was obtained from B.D.H. (Dagenham, UK) and foetal calf serum was obtained from Gibco BRL (UK). Tris-glycine gels (4–12%) were purchased from Novex (British Biotechnology Limited, Oxford, UK). Pure nitrocellulose membrane (0.45 μm) and filter paper were purchased from BioRad (Hertfordshire, UK).

2.7. Statistical analysis

Results are shown as mean ± S.E.M. from triplicate determinations (wells) from 3 separate experimental days ($n = 9$). Student's paired or unpaired t-tests, as appropriate, were used to determine the significance of differences between means and a P-value of less than 0.05 was taken as statistically significant.

3. Results

3.1. Accumulation of cyclooxygenase metabolites from endogenous arachidonic acid by endothelial cells or macrophages incubated with endotoxin

The relatively low levels of cyclooxygenase metabolites released from untreated cells and accumulating in the supernatant after 3 h were not significantly increased after 24 h incubation (Fig. 1; $n = 9$). The addition to the cell cultures of endotoxin, over a range of concentrations from 0.001 to 1 μg ml^{-1}, increased the accumulation of cyclooxygenase metabolites from either cell type in a dose-dependent manner (data not shown). As a result of these initial experiments, a single, fixed concentration of 1 μg ml^{-1} was used in all subsequent work. This concentration did not affect the

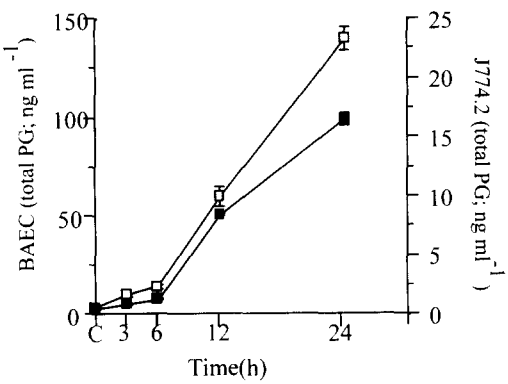

Fig. 1. Increased accumulation of cyclooxygenase metabolites in the culture medium of bovine aortic endothelial cells or murine macrophages incubated with endotoxin (1 μg ml^{-1}) over 24 h. The values shown represent total cyclooxygenase metabolites (6-oxo-prostaglandin $F_{1\alpha}$, prostaglandin E_2, prostaglandin $F_{2\alpha}$ and thromboxane B_2) present at the different times. Untreated cells accumulated very small amounts of cyclooxygenase metabolites over 24 h (C). In the presence of endotoxin, the amounts of cyclooxygenase metabolites in the medium of both bovine aortic endothelial cells (open squares) and macrophages (filled squares) were increased several-fold, although the total output from macrophages was approximately 10-fold less than that from bovine aortic endothelial cells.

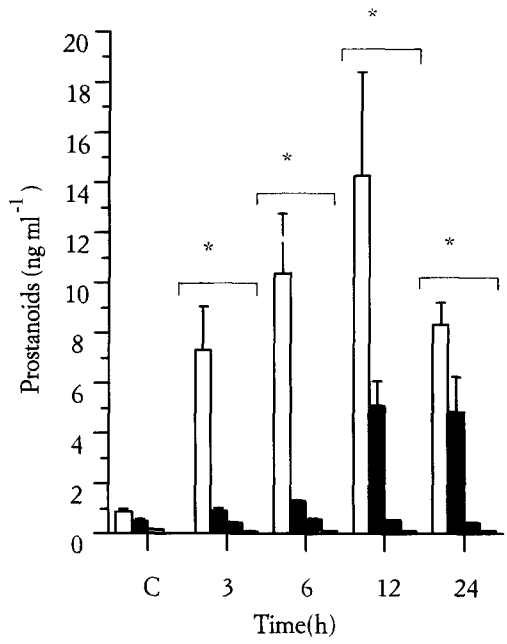

Fig. 2. Time-dependent increase of cyclooxygenase activity in endotoxin-activated bovine aortic endothelial cells measured by the formation of 6-oxo-prostaglandin $F_{1\alpha}$ (open columns), prostaglandin E_2 (filled columns), thromboxane B_2 (hatched columns) and prostaglandin $F_{2\alpha}$ (striped columns) in the presence of exogenous arachidonic acid (30 μM; 15 min). Data are expressed as mean ± S.E.M. from triplicate determinations (wells) from 3 separate experimental days ($n = 9$). * $P < 0.05$ when compared to untreated cells at 24 h (C).

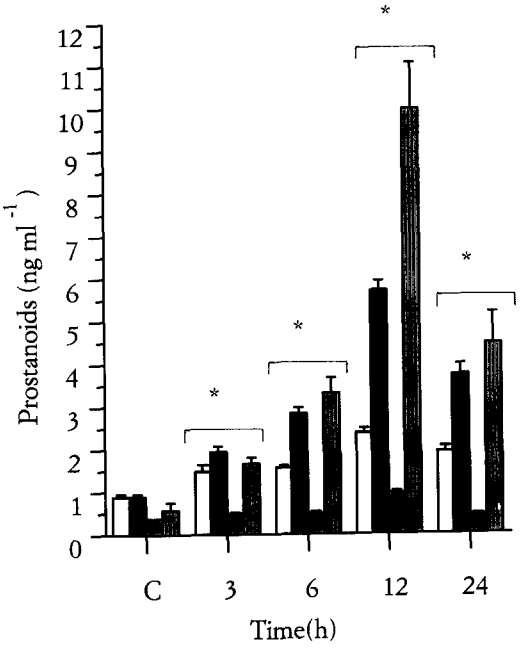

Fig. 3. Time-dependent increase of cyclooxygenase activity in endotoxin-activated murine macrophages measured by the formation of 6-oxo-prostaglandin $F_{1\alpha}$ (open columns), prostaglandin E_2 (filled columns), thromboxane B_2 (hatched columns) and prostaglandin $F_{2\alpha}$ (striped columns) in the presence of exogenous arachidonic acid (30 μM; 15 min). Data are expressed as mean ± S.E.M. from triplicate determinations (wells) from 3 separate experimental days ($n = 9$). * $P < 0.05$ when compared to untreated cells at 24 h (C).

viability of the cells as determined by the MTT assay over 24 h (> 80% viability of control cells).

The effects of adding endotoxin (1 μg ml^{-1}) to the two cell types on the accumulation of cyclooxygenase metabolites over 24 h are shown in Fig. 1. The increase was first significant at 3 h and continued over 24 h. Although the time course of the increase after endotoxin was similar in both cell types, the total amounts of metabolites formed were very different (140 ng ml^{-1} for the bovine aortic endothelial cells, but only 16 ng ml^{-1} for the macrophages). Moreover, the composition of the mixture of cyclooxygenase metabolites was different. At 24 h endothelial cells released predominantly 6-oxo-prostaglandin $F_{1\alpha}$ (94 ± 20 ng ml^{-1}), and to a lesser extent prostaglandin E_2 (42 ± 3 ng ml^{-1}), thromboxane B_2 (1.8 ± 0.2 ng ml^{-1}) and prostaglandin $F_{2\alpha}$ (1.2 ± 0.1 ng ml^{-1}), ($n = 9$). In contrast, macrophages activated with endotoxin produced mainly prostaglandin $F_{2\alpha}$ (7.1 ± 1.6 ng ml^{-1}) and prostaglandin E_2 (5.7 ± 0.2 ng ml^{-1}) and smaller amounts of 6-oxo-prostaglandin $F_{1\alpha}$ (2.6 ± 0.2 ng ml^{-1}) and thromboxane B_2 (1.07 ± 0.02 ng ml^{-1}), ($n = 9$). The rank order of cyclooxygenase metabolites released at 24 h after endotoxin was representative of the release at all time points (data not shown). The release of cyclooxygenase metabolites in both cell types, stimulated by endotoxin, was strongly inhibited (> 75%) by pretreatment with cycloheximide (10 μg ml^{-1}) or dexamethasone (1 μM) and by the addition of acetylsalicylic acid or indomethacin (100 μg ml^{-1}; $n = 9$ for each condition).

3.2. Activity of cyclooxygenase after different times of incubation with endotoxin

We obtained a more direct estimate of cyclooxygenase activity by incubating washed cells with exogenous substrate (30 μM for 15 min) and measuring the cyclooxygenase metabolites formed. From these assays, cyclooxygenase activity was increased by endotoxin treatment for 24 h (Figs. 2 and 3) but for both cell types, the maximum value of cyclooxygenase activity was now attained at 12 h. Cyclooxygenase activity at 24 h was still higher than in untreated cells, but only about 50% of the maximum. Figs. 2 and 3 also show that with exogenous substrate, the major cyclooxygenase metabolites were 6-oxo-prostaglandin $F_{1\alpha}$ for bovine aortic endothelial cells and prostaglandin $F_{2\alpha}$ for macrophages. The stimulation by endotoxin of cyclooxygenase activity measured by this method was much less

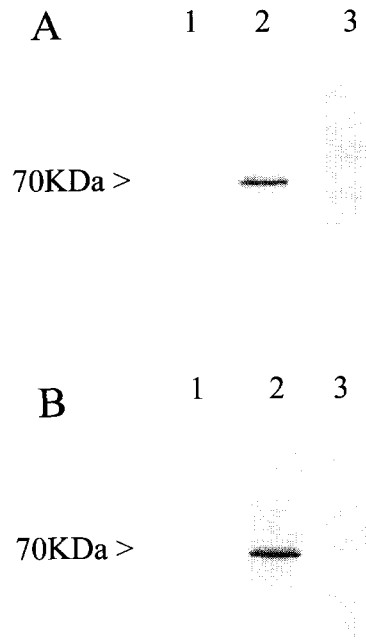

Fig. 4. Western blots using polyclonal antibodies to cyclooxygenase-2 of cell extracts from endotoxin-treated and untreated bovine aortic endothelial cells (panel A) and murine macrophages (panel B). Equal amounts of protein were loaded in all lanes of bovine aortic endothelial cells (1–2 μg/lane) and macrophages (4–5 μg/lane). Control untreated bovine aortic endothelial cells or macrophages (lane 1) contained no cyclooxygenase-2 protein. In contrast, endotoxin-activated (1 μg ml^{-1} for 24 h) bovine aortic endothelial cells or macrophages contained cyclooxygenase-2 protein (lane 2). The induction of cyclooxygenase-2 protein by endotoxin in both bovine aortic endothelial cells and macrophages was abolished by cycloheximide (10 μg ml^{-1}; 30 min prior to endotoxin; lane 3). Similar results were obtained using cell extracts from 3 separate batches of cells.

(> 80% inhibition) after pretreatment of either cell type with cycloheximide (10 μg ml^{-1}). Pretreatment with dexamethasone (1 μM) was equally effective in decreasing the stimulated cyclooxygenase activity in macrophages (> 80% inhibition), but less so in bovine aortic endothelial cells (about 40% inhibition; $n = 9$).

3.3. Characterisation of cyclooxygenase isoforms present in endothelial cells and macrophages

Extracts of untreated bovine aortic endothelial cells or macrophages contained no detectable cyclooxygenase-2 protein, as determined by Western blot analysis (Fig. 4; detectable level is 1–10 ng of protein). In contrast, after incubation for 24 h with endotoxin, bovine aortic endothelial cells or macrophages contained a protein of approximately 70 kDa, which was recognised by a specific antibody to cyclooxygenase-2 (Fig. 4). This figure also shows that the degree of induction of cyclooxygenase-2 protein caused by endotoxin in endothelial cells was comparable to that elicited by endotoxin in macrophages. As shown in lane 3 of the figure, the induction of cyclooxygenase-2 protein by endotoxin in both endothelial cells and macrophages was abolished by pretreatment of the cells with cycloheximide (10 μg ml^{-1}).

3.4. Accumulation of nitrite in the medium of endothelial cells and macrophages after incubation with endotoxin

Incubation with endotoxin (1 μg ml^{-1}) caused a time-dependent increase in nitrite accumulation in the culture medium of macrophages after 6 and up to 24 h (Fig. 5; $n = 9$). This formation of nitrite after endotoxin was inhibited by more than 80% by the NOS inhibitor, N^G-monomethyl-L-arginine (1 mM; administered together with endotoxin, $n = 9$). Cycloheximide (10 μg ml^{-1}; 30 min prior to endotoxin) or dexamethasone (1 μM; 1 h prior to endotoxin) also prevented the increased nitrite release in macrophages stimulated by incubation with endotoxin (Fig. 5; $n = 9$).

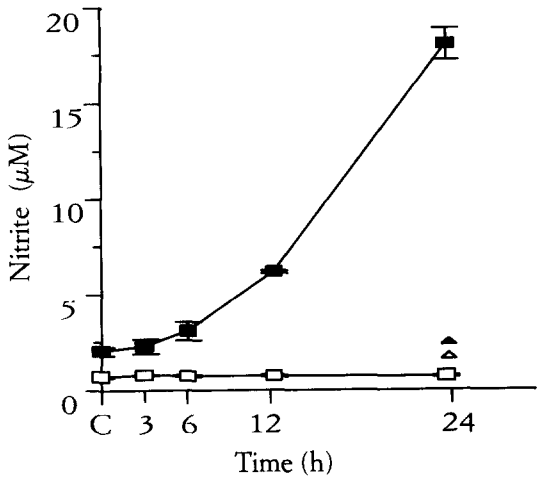

Fig. 5. Time-course for the accumulation of nitrite in the culture medium of bovine aortic endothelial cells (open squares) and macrophages (closed squares) stimulated with endotoxin. Data are expressed as mean ± S.E.M. from triplicate determinations (wells) from 3 separate experimental days ($n = 9$). * $P < 0.05$ when compared to untreated cells at 24 h (C). The open triangle represents the concentration of nitrite in the medium of macrophages treated with endotoxin plus dexamethasone (1 μM; 1 h prior to endotoxin). The closed triangle represents the nitrite concentration in the culture medium of macrophages treated with endotoxin plus cycloheximide (10 mg μl^{-1}; 30 min prior to endotoxin).

In contrast to macrophages, bovine aortic endothelial cells did not release detectable amounts (< 1 μM) of nitrite when stimulated with endotoxin (1 μg ml^{-1}) for up to 24 h (Fig. 5; $n = 9$). Addition of acetylsalicylic acid or indomethacin (either at 100 μg ml^{-1}) had no significant effect on nitrite formation from bovine aortic endothelial cells over 24 h with or without endotoxin ($n = 9$; data not shown).

4. Discussion

Here we show that incubation of bovine aortic endothelial cells or macrophages with endotoxin for 24 h results in (i) accumulation of cyclooxygenase metabolites, (ii) enhanced cyclooxygenase activity, and (iii) induction of cyclooxygenase-2 protein. The increased accumulation of cyclooxygenase metabolites derived from endogenous arachidonic acid could result from an increased availability of endogenous arachidonic acid due to increased activity of phospholipase A_2 (Glaser et al., 1993). However, endotoxin also increased the formation of cyclooxygenase metabolites from exogenous substrate, implying an increase in cyclooxygenase enzymatic activity. Increased cyclooxygenase activity in macrophages after endotoxin treatment has already been noted (Mitchell et al., 1993), but a comparison with the increased activity in bovine aortic endothelial cells under similar conditions shown here disclosed two interesting discrepancies. First, although the accumulated amounts of cyclooxygenase metabolite formed from endogenous substrate were highest at 24 h, the maximal level of cyclooxygenase activity exhibited towards exogenous substrate was reached at 12 h with a clear fall at 24 h. This difference could reflect the stability of the accumulated cyclooxygenase metabolites in the supernatant medium (unpublished experiments showed no loss over 12 h of exogenous prostaglandin E_2 added to cultures of either cell type). At longer incubation times, for instance 36 h, a fall in accumulated metabolites might have been shown. It would also be compatible with the shorter half-life of cyclooxygenase-2 mRNA and protein (relative to that of cyclooxygenase-1) observed in other systems (Nusing and Ullrich, 1992; Hamasaki et al., 1993).

Second, after 12 h of incubation with endotoxin, the accumulated metabolites in bovine aortic endothelial

cell cultures were 6 times greater than in macrophages, but the level of cyclooxygenase activity towards exogenous arachidonic acid in the two cell types was essentially equal. This equivalence was matched by the equal increase of cyclooxygenase-2 protein shown by the immunoblot assay in both cell types. We have already reported the induction of cyclooxygenase-2 protein in macrophages (Mitchell et al., 1993) and here we show that cyclooxygenase-2 is induced by endotoxin also in bovine aortic endothelial cells.

Thus, the increased cyclooxygenase activity exhibited by the cells was reflected by the induction of cyclooxygenase-2 protein. Although the cyclooxygenase-1 isoform is also known to be increased after a variety of stimuli (Hamasaki et al., 1993; Hoff et al., 1993; Smith et al., 1993) the magnitude of the increase and its susceptibility to inhibition by dexamethasone are less than that found with cyclooxygenase-2 (Hoff et al., 1993; O'Banion et al., 1992; Kujubu and Herschman, 1992). Another possible contributory factor which would be affected by dexamethasone is the provision of endogenous substrate by phospholipid hydrolysis. There is indirect evidence for increased levels of endogenous arachidonic acid contributing to accumulation of 6-oxo-prostaglandin $F_{1\alpha}$ in smooth muscle cells following interleukin-1 and more directly, the mRNA for phospholipase A_2 was increased in endothelial cells exposed to interleukin-1 (Jackson et al., 1993), a cytokine released from endothelial cells by endotoxin (Mantovani et al., 1992). Such an increase of phospholipase A_2 activity in endothelial cells, but not in macrophages, after exposure to endotoxin could account for the 10-fold higher accumulation of cyclooxygenase metabolites from endogenous substrate in bovine aortic endothelial cell cultures, although synthesis from exogenous arachidonic acid was approximately equal, as was the amount of cyclooxygenase-2 enzyme induced.

Although the induction of cyclooxygenase-2 in the two cell types was similar, the effects of endotoxin on inducible NO synthase were substantially different with a more than 10-fold increase in nitrite production in the macrophages and no detectable increase in bovine aortic endothelial cell cultures. Increased inducible NO synthase activity following endotoxin exposure in macrophages is a well-established phenomenon (Di Rosa et al., 1990; McCall et al., 1991; Baydoun et al., 1994) and in our experiments it served as a positive control for the effects of endotoxin. The lack of increased inducible NO synthase activity in the bovine aortic endothelial cells must be considered in context. Firstly the assay we used, the Griess reaction, has a detection limit of 1 μM nitrite and this is clearly not able to measure NO production by bovine aortic endothelial cells which may be detectable by bioassay on smooth muscle or on platelets or by increases in cGMP (Gryglewski et al., 1986; Salvemini et al., 1990; Lamas et al., 1991). Radomski et al. (1990), using the oxyhaemoglobin assay, found NO formed from their endothelial cell preparation was about 100 pmol NO/mg microsomal protein. After endotoxin, this was increased by 10 pmol/mg; even after treatment with endotoxin and interferon-γ, the additional activity was only 40 pmol/mg, a 40% increase over the basal value. We would not have been able to detect this level of activity or this magnitude of change by the Griess assays. Thus, we cannot demonstrate conclusively that NO synthase in the bovine aortic endothelial cells was not increased at all; any such increase was very clearly less than that observed in the macrophages over 24 h with endotoxin. However, there is clear evidence of the lack of induction of inducible NO synthase in human (MacNual and Hutchinson, 1993) or rat (Marumo et al., 1993) aortic endothelial cells, following treatment with endotoxin or cytokines. Further, no increase in nitrite accumulation was reported in bovine aortic endothelial cells cultures incubated with endotoxin for 24 h, using the sensitive chemiluminesence assay (Berman et al., 1993). It is possible that a low level of induction of inducible NO synthase, relative to the macrophage, is a general characteristic of aortic endothelia. There may be a pathophysiological purpose to this differential induction. High concentrations of NO are considered to be cytotoxic (Moncada et al., 1991) and as such would be appropriate in conditions where activated macrophages with induced NO synthase may be present (inflammation, infection and other host defence conditions). Even in these situations, however, cytotoxic behaviour would not be helpful at the level of the endothelium, a cell that needs to be protected especially in conditions where oxygen derived or other free radicals are being generated.

What, then, is the mechanism by which endotoxin greatly increased NO synthase activity in the murine macrophages, but not in bovine aortic endothelial cells? One possibility is the inhibition of NO synthase by cyclooxygenase metabolites. Exogenous prostaglandin E_2 or the prostacyclin analogue, iloprost, inhibit the induction of inducible NO synthase activity in endotoxin-activated macrophages (Marotta et al., 1992). In our experiments, endotoxin treatment of bovine aortic endothelial cells released large amounts of prostaglandin E_2 and prostaglandin I_2 and these may have attenuated the increase of NO synthase activity in these cells. This possibility, however, was excluded, because acetylsalicylic acid or indomethacin, in amounts sufficient to inhibit the accumulation of cyclooxygenase metabolites by bovine aortic endothelial cells, did not alter the amounts of NO produced. Another possibility is that bovine aortic endothelial cells release a factor(s) which permits increased cyclooxygenase activity, but selectively suppresses NO synthase induction or activity. Endothelial cells are known to release several

mediators including platelet derived growth factor (Cross and Dexter, 1991) when stimulated by interleukin-1 (Mantovani et al., 1992) and tumour necrosis factor (Puztai et al., 1993): interleukin-1 and tumour necrosis factor are both evoked by endotoxin. As platelet derived growth factor inhibits the induction of inducible NO synthase (Schini et al., 1992) and induces cyclooxygenase-2 activity (Rothe and Falanga, 1989), an enhanced formation of platelet derived growth factor may well attenuate any potential induction of inducible NO synthase in bovine aortic endothelial cells activated with endotoxin.

It is also possible that endotoxin needs the presence of other cytokines to induce inducible NO synthase activity in bovine aortic endothelial cells. In vivo, endotoxin may generate these other factors from a variety of cell types, directly or indirectly, but in a monoculture these additional factors have to be provided. Thus, endotoxin in combination with other cytokines induced inducible NO synthase activity in murine endothelial cells (Kilbourn and Belloni, 1990; Gross et al., 1991). However, endotoxin alone did not induce nitrite production (Oswald et al., 1994) or mRNA for inducible NO synthase (Marumo et al., 1993) in endothelial cell cultures, where mixtures of interferon-γ with tumour necrosis factor-α or endotoxin or other cytokines were highly effective. Nevertheless, if a mixture of cytokines is needed then the cytokines essential to the induction of inducible NO synthase in bovine aortic endothelial cells must be different from those required by the macrophages. The elucidation of these cytokines and their transduction pathways which lead to the induction of inducible NO synthase and cyclooxygenase-2 in a variety of cells, will help to identify differences that may be turned to therapeutic advantage.

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References

Akarasereenont, P., J.A. Mitchell, C. Thiemermann and J.R. Vane, 1994, Lipopolysaccharide induces bovine aortic endothelial cells to synthesize cyclooxygenase but not nitric oxide synthase: a comparison with macrophages, Br. J. Pharmacol. 111 (Suppl.), 33P.

Baydoun, A.R., R.G. Bogle, J.D. Pearson and G.E. Mann, 1993, Selective inhibition by dexamethasone of induction of NO synthase, but not of induction of L-arginine transport, in activated murine macrophage J774 cells, Br. J. Pharmacol. 110, 1401.

Berman, R.S., J.D. Frew and W. Martin, 1993, Endotoxin-induced arterial endothelial barrier dysfunction assessed by an in vivo model, Br. J. Pharmacol. 110, 1282.

Cross, M. and T.M. Dexter, 1991, Growth factors in development, transformation, and tumorigenesis, Cell 64, 271.

De Nucci, G., R.J. Gryglewski, T.D. Warner and J.R. Vane, 1988, Receptor mediated release of endothelium-derived relaxing factor and prostacyclin from bovine aortic endothelial cells is coupled, Proc. Natl. Acad. Sci. USA 85, 2334.

Di Rosa, M., M.W. Radomski, R. Carnuccio and S. Moncada, 1990, Glucocorticoids inhibit the induction of nitric oxide synthase in macrophages, Biochem. Biophys. Res. Commun. 172, 1246.

Glaser, K.B., D. Mobilio, J.Y. Chang and N. Senko, 1993, Phospholipase A_2 enzymes: regulation and inhibition, Trends Pharmacol Sci. 14, 92.

Gross, S.S. and R. Levi, 1992, Tetrahydrobiopterin synthesis: an absolute requirement for cytokine-induced nitric oxide generation by vascular smooth muscle, J. Biol. Chem. 267, 25722.

Gross, S.S., E.A. Jaffe, R. Levi and R.G. Kilbourn, 1991, Cytokine-activated endothelial cells express an isotype of nitric oxide synthase which is tetrahydrobiopterin dependent, calmodulin-independent and inhibited by arginine analogs with a rank-order of potency characteristic of activated macrophages, Biochem. Biophys. Res. Commun. 178, 823.

Gryglewski, R.J., S. Moncada and R.J. Palmer, 1986, Bioassay of prostacyclin and endothelium-derived relaxing factor (EDRF) from porcine aortic endothelial cells, Br. J. Pharmacol. 87, 685.

Hamasaki, Y., J. Kitzler, R. Hardman, P. Nettesheim and T.E. Eling, 1993, Phorbol ester and epidermal growth factor enhance the expression of two inducible prostaglandin H synthase genes in rat tracheal epithelial cells, Arch. Biochem. Biophys. 304, 226.

Hoff, T., D. De Witt, V. Kaever, K. Resch and M. Goppelt-Strueebe, 1993, Differentiation-associated expression of prostaglandin G/H synthase in monocytic cells, FEBS Lett. 320, 38.

Jackson, B.A., R.H. Goldstein, R. Roy, M. Cozzani, L. Taylor and P. Polgar, 1993, Effects of transforming growth factor-β and interleukin-1β on expression of cyclooxygenase 1 and 2 and phospholipase A_2 mRNA in lung fibroblasts and endothelial cells in culture, Biochem. Biophys. Res. Commun. 197, 1465.

Kilbourn, R.G. and P. Belloni, 1990, Endothelial cell production of nitrogen oxides in response to interferon γ in combination with tumor necrosis factor, interleukin 1, or endotoxin, J. Natl. Cancer Inst. 82, 772.

Kujubu, D.A. and H.R. Herschman, 1992, Dexamethasone inhibits mitogen induction of the TIS10 prostaglandin synthase/cyclooxygenase gene, J. Biol. Chem. 267, 7991.

Lamas, S., T. Michel, B.M. Brenner and P.A. Marsden, 1991, Nitric oxide synthesis in endothelial cells: evidence for a pathway inducible by TNF-α, Am. J. Physiol. 261, C634.

Lee, S.H., E. Soyoola, P. Chanmugam, S. Hart, W. Sun, H. Zhong, S. Liou, D. Simmons and D. Hwang, 1992, Selective expression of mitogen inducible cyclo-oxygenase in macrophages stimulated with lipopolysaccharide, J. Biol. Chem. 267, 25934.

MacNaul, K.L. and N.L. Hutchinson, 1993, Differential expression of iNOS and cNOS mRNA in human vascular smooth muscle cells and endothelial cells under normal and inflammatory conditions, Biochem. Biophys. Res. Commun. 196, 1330.

Mantovani, A., F. Bussolino and E. Dejana, 1992, Cytokine regulation of endothelial cell function, FASEB J. 6, 2591.

Marotta, P., L. Sautebin and M. Di Rosa, 1992, Modulation of the induction of nitric oxide synthase by eicosanoids in the murine macrophage cell line J774, Br. J. Pharmacol. 107, 640.

Marumo, T., T. Nakaki, H. Adachi, H. Esumi, H. Suzuki, T. Saruta and R. Kato, 1993, Nitric oxide synthase mRNA in endothelial cells: synergistic induction by interferon-γ, tumor necrosis factor-α and lipopolysaccharide and inhibition by dexamethasone, Jpn. J. Pharmacol. 63, 327.

McCall, T.B., M. Feelisch, R.M.J. Palmer and S. Moncada, 1991, Identification of *N*-iminoethyl-L-ornithine as an irreversible inhibitor of nitric oxide synthase in phagocytic cells, Br. J. Pharmacol. 102, 234.

Mitchell, J.A., P. Akarasereenont, C. Thiemermann, R.J. Flower and J.R. Vane, 1993, Selectivity of nonsteroidal antiinflammatory drugs as inhibitors of constitutive and inducible cyclooxygenase, Proc. Natl. Acad. Sci. USA 90, 11693.

Moncada, S., R.M.J. Palmer and E.A. Higgs, 1991, Nitric oxide: physiology, pathophysiology, and pharmacology, Pharmacol. Rev. 43, 109.

Nusing, R. and V. Ullrich, 1992, Regulation of cyclooxygenase and thromboxane synthase in human monocytes, Eur. J. Biochem. 206, 131.

O'Banion, M.K., V.D. Winn and D.A. Young, 1992, cDNA cloning and functional activity of a glucocorticoid-regulated inflammatory cyclooxygenase, Proc. Natl. Acad. Sci. USA 89, 4888.

Oswald, I.P., I. Eltoum, T.A. Wynn, B. Schwartz, P. Caspar, D. Paulin, A. Sher and S.L. James, 1994, Endothelial cells are activated by cytokine treatment to kill an intravascular parasite, *Schistosoma mansoni*, through the production of nitric oxide, Proc. Natl. Acad. Sci. USA 91, 999.

Pusztai, L., C.E. Lewis, J. Lorenzen and J.O.D. McGee, 1993, Growth factors: regulation of normal and neoplastic growth, J. Pathol. 169, 191.

Radomski, M.W., R.M.J. Palmer and S. Moncada, 1990, Glucocorticoids inhibit the expression of an inducible, but not the constitutive, nitric oxide synthase in vascular endothelial cells, Proc. Natl. Acad. Sci. USA 87, 10043.

Rothe, M. and V. Falanga, 1989, Growth factors: their biology and promise in dermatologic disease and tissue repair, Arch. Dermatol. 125, 1390.

Salmon, J.A., 1978, A radioimmunoassay for 6-keto-prostaglandin $F_{1\alpha}$, Prostaglandins 15, 383.

Salvemini, D., R. Korbut, E. Änggård and J.R. Vane, 1990, Immediate release of a nitric oxide-like factor from bovine aortic endothelial cells by *Escherichia coli* lipopolysaccharide, Proc. Natl. Acad. Sci. USA 87, 2593.

Salvemini, D., T.P. Misko, J.L. Masferrer, K. Seibert, M.G. Currie and P. Needleman, 1993, Nitric oxide activates cyclooxygenase enzymes, Proc. Natl. Acad. Sci. USA 90, 7240.

Schini, V.B., W. Durante, E. Elizondo, T. Scott-Burden, D.C. Junqnero, A.I. Schafer and P.M. Vanhoutte, 1992, The induction of nitric oxide synthase activity is inhibited by TGF-β_1, $PDGF_{AB}$ and $PDGF_{BB}$ in vascular smooth muscle cells, Eur. J. Pharmacol. 216, 379.

Smith, W.L. and L.J. Marnett, 1991, Prostaglandin endoperoxide synthase: structure and catalysis, Biochim. Biophys. Acta 1083, 1.

Smith, C.J., J.D. Morrow, L.J. Roberts and L.J. Marnett, 1993, Differentiation of monocytoid THP-1 cells with phorbol ester induces expression of prostaglandin endoperoxide synthase-1 (COX-1), Biochem. Biophys. Res. Commun. 192, 787.

Thiemermann, C., 1994, The role of arginine: nitric oxide pathway in circulatory shock, Adv. Pharmacol. 28, 45.

Vane, J.R. and R.M. Botting, 1990, Mediators from the endothelial cell, Adv. Pros. Thromb. Leuk. Res. 21, 627.

Vane, J.R. and R.M. Botting, 1992, The prostaglandins, in: Aspirin and other Salicylates, eds. J.R. Vane and R.M. Botting (Chapman & Hall, London) p. 17.

Xie, W., D.L. Robertson and D.L. Simmons, 1992, Mitogen-inducible prostaglandin G/H synthase: a new target for nonsteroidal anti-inflammatory drugs, Drug Dev. Res. 25, 249.