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A771726, the active metabolite of leflunomide, directly inhibits the activity of cyclo-oxygenase-2 *in vitro* and *in vivo* in a substrate-sensitive manner

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1 The immunosuppressive and anti-inflammatory drug leflunomide has several sites of action, although its precise mode of action is unknown.

2 Here we show *in vitro* and *in vivo* that leflunomide and/or its active metabolite A771726, inhibit the activity of cyclo-oxygenase (COX) at doses below those that affect protein expression.

3 In J774.2 macrophages treated with endotoxin for 24 h to induce COX-2 and iNOS, leflunomide and A771726 inhibited more potently the accumulation of PGE_2 (A771726, IC_{50} 3.5 $\mu g\ ml^{-1}$) than of NO_2 (A771726, IC_{50} 380 $\mu g\ ml^{-1}$). At high concentrations ($>300\ \mu g\ ml^{-1}$) A771726 also exhibited the expression of COX-2 and iNOS proteins.

4 In A549 cells treated for 24 h with interleukin-1β, to induce COX-2, A771726 potently inhibited PGE_2 synthesis (IC_{50} 0.13 $\mu g\ ml^{-1}$). In the same cells, A771726 was notably less active (IC_{50}, 52 $\mu g\ ml^{-1}$) at inhibiting the formation of PGE_2 stimulated by exposure to 30 μM arachidonic acid.

5 In a human whole blood assay, measuring the accumulation of TxB_2 in response to calcium ionophore as a measure of COX-1 activity and in response to incubation with bacterial endotoxin as a measure of COX-2 activity, leflunomide inhibited COX-1 and COX-2 with IC_{50} values of 31 and 185 $\mu g\ ml^{-1}$; for A771726 the corresponding values were 40 and 69 $\mu g\ ml^{-1}$.

6 Pre-treatment of rats with leflunomide or A771726 (10 mg kg^{-1}, i.p.) inhibited the plasma accumulation of 6-keto-$PGF_{1\alpha}$ but not NO_2/NO_3 following infusion of endotoxin. Injection of a bolus of arachidonic acid following 6 h infusion of endotoxin caused a marked acute rise in plasma 6-keto-$PGF_{1\alpha}$ which was inhibited only by higher doses of A771726 (50 mg kg^{-1}, i.p.).

7 In conclusion, leflunomide *via* A771726 can directly inhibit the activity of COX, an effect that appears blunted both by increases in substrate supply and possibly by plasma binding. Only at much higher drug levels does leflunomide and/or A771726 inhibit the induction of COX-2 or iNOS proteins.

Keywords: Cyclo-oxygenase; nitric oxide synthase; leflunomide; nonsteroidal anti-inflammatory drugs; arachidonic acid

Abbreviations: COX, cyclo-oxygenase; LPS, lipopolysaccharide; NOS, nitric oxide synthase; NSAIDs, non-steroidal anti-inflammatory drugs

Introduction

Leflunomide (N-(4-triflurormethylphenyl)-5-methylisoxazol-4-carboxamide) is a novel immunomodulating drug which is of interest for the treatment of the chronic inflammatory condition, rheumatoid arthritis (Mladenovic, 1995; Silva & Morris, 1997). It is a pro-drug, exerting its therapeutic effects primarily through A771726 (N-(4-triflurormethylphenyl)-2-cyano-3-hydroxy-crotonic acid amide), the major metabolite. Depite its biochemical mode of action being largely unknown, leflunomide appears to have several sites of action (Silva & Morris, 1997). In addition to reducing local concentrations of inflammatory mediators, inhibiting, for example, the release of histamine from mast cells and the release of reactive oxygen species from white blood cells (Bartlett *et al*., 1993), leflunomide also interferes with the activation, proliferation and differentiation of lymphocytes (Bartlett *et al*., 1991). Leflunomide may also produce anti-proliferative effects by inhibiting the action of dihydroorotate dehydrogenase and so reducing *de novo* pyrimidine biosynthesis. Finally, A771726 may act as an inhibitor of certain tyrosine kinases (Mattar *et al*., 1993).

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Inflammation is associated with the induction of the enzyme cyclo-oxygenase-2 (COX-2) (Hla *et al*., 1993; Mitchell *et al*., 1994; Seibert & Masferrer, 1994; Tomlinson *et al*., 1994; Vane *et al*., 1994). COX-2 is the mitogen-inducible isoform of the 'house-keeping' enzyme COX-1 (Xie *et al*., 1991), which controls the synthesis of prostaglandins that contribute to normal cell activity (Vane, 1971). Both COX-1 and COX-2 are pharmacological targets of the non-steroidal anti-inflammatory drugs (NSAIDs). In particular, inhibition of COX-2 activity may well mediate the therapeutic benefits of these drugs, while inhibition of COX-1 may underlie their harmful side-effects (Vane *et al*., 1994). Many NSAIDs inhibit COX activity by competing with the substrate arachidonic acid for the active site. Consequently, it has been demonstrated both *in vitro* (Laneuville *et al*., 1994; Saunders *et al*., 1996; Mitchell *et al*., 1997) and *in vivo* (Hamilton *et al*., 1999) that depending upon the nature of their binding to the COX enzyme(s) (Gierse *et al*., 1999) NSAID potency is influenced by the supply of arachidonic acid.

Nitric oxide synthase (iNOS) is also induced by inflammatory stimuli (Lorsbach *et al*., 1993; Steur & Marletta, 1989). This enzyme catalyses the conversion of L-arginine to L-citrulline, with the release of nitric oxide (NO). iNOS does not

require calcium as a co-factor for its activity (Xie *et al*., 1992), a feature which differentiates it from the constitutive isoforms of the enzyme, endothelial NOS (eNOS) and neuronal NOS (nNOS). Consequently, iNOS can produce NO in large, cytotoxic quantities, and has therefore been implicated in the oedema, pain, erythema and vasodilatation associated with inflammation (Vane *et al*., 1994).

Here we have tested, *in vitro* and *in vivo*, the hypothesis that leflunomide through the action of A771726 can directly inhibit the activity of COX-2, at concentrations below those required to affect the expression of either COX-2 or iNOS proteins. Some of this data has been presented previously in abstract form to the British Pharmacological Society (Hamilton *et al*., 1997).

Methods

Cell culture

Murine J774.2 macrophages were obtained from the European Collection of Animal Cell Culture (Salisbury, U.K.) and cultured to confluence in 96-well plates, in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% foetal calf serum and 2 mM L-glutamine. Cells were exposed to lipopolysaccharide (LPS, serotype 0127 : B8; 10 μg ml^{-1}) for 24 h at 37°C in the presence or absence of leflunomide (0.003 – 30 μg ml^{-1}), A771726 (0.3 – 1000 μg ml^{-1}), or vehicle (0.1% DMSO for leflunomide, saline for A771726). The human pulmonary epithelial cancer cell line (A549) was also obtained from the European Collection of Animal Cell Culture (Salisbury, U.K.) and cultured in 96-well plates, until confluent, in DMEM containing 10% foetal calf serum and 4 mM L-glutamine. A549 cells were exposed to IL-1β (10 ng ml^{-1}) for 24 h at 37°C in the presence or absence of leflunomide (0.003 – 30 μg ml^{-1}), A771726 (0.3 – 1000 μg ml^{-1}), or vehicle (0.1% DMSO for leflunomide, saline for A771726). In separate experiments, A549 cells were exposed to IL-1β (10 ng ml^{-1}) for 24 h to induce COX-2. Medium was then replaced with fresh medium containing leflunomide (0.003 – 30 μg ml^{-1}), A771726 (0.3 – 1000 μg ml^{-1}), or vehicle (0.1% DMSO for leflunomide, saline for A771726) plus arachidonic acid (30 μM) for 30 min and the medium removed for the measurement of PGE_2.

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-diphenyl-tetrazolium bromide to formazan (Mitchell *et al*., 1993).

Surgical procedure

Male Wistar rats (220 – 250 g; Tuck, U.K.) were anaesthetized with thiobutabarbital sodium (Inactin; 120 mg kg^{-1}, i.p.). The trachea was cannulated to facilitate respiration. The right carotid artery was cannulated and connected to a pressure transducer (Elcomatic Type 750) for the measurement of systemic blood pressure which was recorded on a Graphtec Linearcorder (Type WR 3101). Mean arterial pressure (MAP) was calculated as the diastolic pressure plus one-third of the pulse pressure. Haemodynamic parameters were measured throughout the protocol. A cannula was also introduced into the left jugular vein for the administration of drugs. Body temperature was maintained at 37°C *via* a homeothermic blanket regulated by a rectal thermometer (Biosciences, Sherness, Kent, U.K.).

Upon completion of the surgical procedure, animals were left for 15 min to allow stabilization of the cardiovascular parameters. Animals were treated ($t = -1$ h) with one of the following: leflunomide (20 mg kg^{-1} i.p., $n = 7$), A771726 (10 or 50 mg kg^{-1} i.p., $n = 5-6$), or drug vehicle (DMSO, 10% w v^{-1}, $n = 8$). Commencing 1 h later ($t = 0$) animals received a 6 h continuous infusion of LPS (serotype 0127 : B8, 0.2 mg kg^{-1} h^{-1}) or LPS vehicle (saline). At the end of the infusion period with LPS or vehicle ($t = 6$ h) animals were challenged with a final bolus of arachidonic acid (3 mg kg^{-1}, i.v.). Blood samples (500 μl) were taken *via* the carotid artery cannula at $t = 0$, 2, 4 and 6 h, and 1 min after administration of the final bolus of arachidonic acid.

Measurement of NOS activity

As a measure of NO formation plasma total concentrations of nitrite/nitrate (NO_2/NO_3) were determined. Briefly, NO_3 present within plasma was stoichiometrically reduced to NO_2, by incubation (15 min, 37°C) of samples (10 μl) with nitrate reductase (1 i.u. ml^{-1}), NADPH (500 μM) and flavine adenine dinucleotide (FAD, 50 μM) (final volume 80 μl). Following NO_3 reduction, unused NADPH was oxidized by addition of lactate dehydrogenase (100 i.u. ml^{-1}) and sodium pyruvate (10 mM) (final volume 100 μl) and incubation for 5 min at 37°C. Total NO_2 concentration was assayed by adding 100 μl of Griess reagent (4% sulphanilamide and 0.2% naphtylenediamide in 10% phosphoric acid) to each 100 μl sample which forms a purple azo dye in the presence of nitrite. The formation of this dye was measured spectrophotometrically at 550 nm with a reference filter at 650 nm (Molecular Devices, Richmond, CA, U.S.A.). Total NO_2 concentrations (nmol ml^{-1}) were calculated by comparison with the optical density of standard solutions of sodium nitrite and sodium nitrate (also stochiometrically reduced to NO_2) prepared in plasma. NO accumulates in culture medium as NO_2. 100 μl of culture medium was added to 100 μl of Griess reagent in 96 well plates. The absorbance was read as described above.

Measurement of COX activity

As an indicator of COX activity the accumulation of PGE_2 in the culture medium was measured by specific radioimmunoassay. The assay detection limit was approximately 10 pg per tube. *In vivo*, the plasma concentration of 6 keto-prostaglandin (PG) $F_{1\alpha}$, the stable hydrolysis product of prostacyclin (PGI_2), was measured by specific radioimmunoassay as a determinant of COX activity. The percentage cross reactivity (at 50% displacement) of the antibody against 6-keto-$PGF_{1\alpha}$ used in this study against other eicosanoids was the following: PGE_2 11%; $PGF_{2\alpha}$ 10%; thromboxane B_2 (TXB_2) 0.05%. The detection limit of this assay is approximately 10 pg per tube with an inter-assay variability of 7%.

Human whole blood assay

Blood was collected by venupuncture into heparin (19 U ml^{-1}) and then aliquoted in 100 μl volumes into the individual wells of 96-well plates. For COX-1 assays, blood was then treated with leflunomide or A771726 or vehicle, followed 60 min later by calcium ionophore, A23187 (50 μM). After 30 min the plates were centrifuged (1500 $\times g$, 4°C, 5 min) and the plasma removed and immediately frozen. For COX-2 assays, blood was treated with aspirin (12 μg ml^{-1}) to inactivate COX-1, and then 6 h later with bacterial endotoxin (LPS, 10 μg ml^{-1}) plus test agents or vehicle. Incubation was then continued for a

further 18 h, after which time the plates were spun and the plasma removed and frozen. Concentrations of thromboxane (Tx) B_2 (as a measure of TxA_2 formation and so COX activity) in samples from both protocols were then determined by radioimmunoassay.

Western blot analysis

Induction of iNOS and COX-2 in J774 macrophages following stimulation with LPS was determined by Western blot analysis using specific polyclonal antibodies raised to iNOS and COX-2 proteins (Hamilton & Warner, 1998).

Materials

[^{3}H] 6-Keto-$PGF_{1\alpha}$ and [^{3}H] PGE_2 were bought from Amersham International (Little Chalfont, Bucks., U.K.). Leflunomide and A771726 were generous gifts from Dr A. Bartlett, Hoescht (Germany). The COX-2-selective antibody was a gift from Merck-Frosst (Canada) and the iNOS selective antibody was a gift from Dr C. Byrant (Cambridge, U.K.). All other compounds were purchased from Sigma Chemical Company (Poole, Dorset, U.K.).

Results

Stimulation of COX-2 and iNOS activity in J774.2 macrophages: effect of leflunomide and A771726

Incubation of J774.2 macrophages with LPS for 24 h increased the accumulation, in the medium, of both PGE_2 and NO_2, 13.1 ± 0.9 ng ml^{-1} and 40 ± 7 μM respectively. Treatment of cells with either leflunomide or A771726 resulted in a concentration-dependent reduction in PGE_2 accumulation, with IC_{50} values of 2.2 and 3.5 μg ml^{-1}, respectively ($n = 9-12$; Figure 1). A771726 inhibited the accumulation of NO_2 only at much higher concentrations (IC_{50} 380 μg ml^{-1}, $n = 9-12$) (Figure 1). Due to its limited solubility, leflunomide could not be administered at sufficient concentrations to permit calculation of an IC_{50} value for the inhibition of NO_2 formation.

Expression of iNOS and COX-2 in J774.2 macrophages

The rise in PGE_2 and NO_2 synthesis in LPS-treated J774.2 macrophages was associated with the induction of iNOS and COX-2 protein, as confirmed by Western blot analysis (Figure

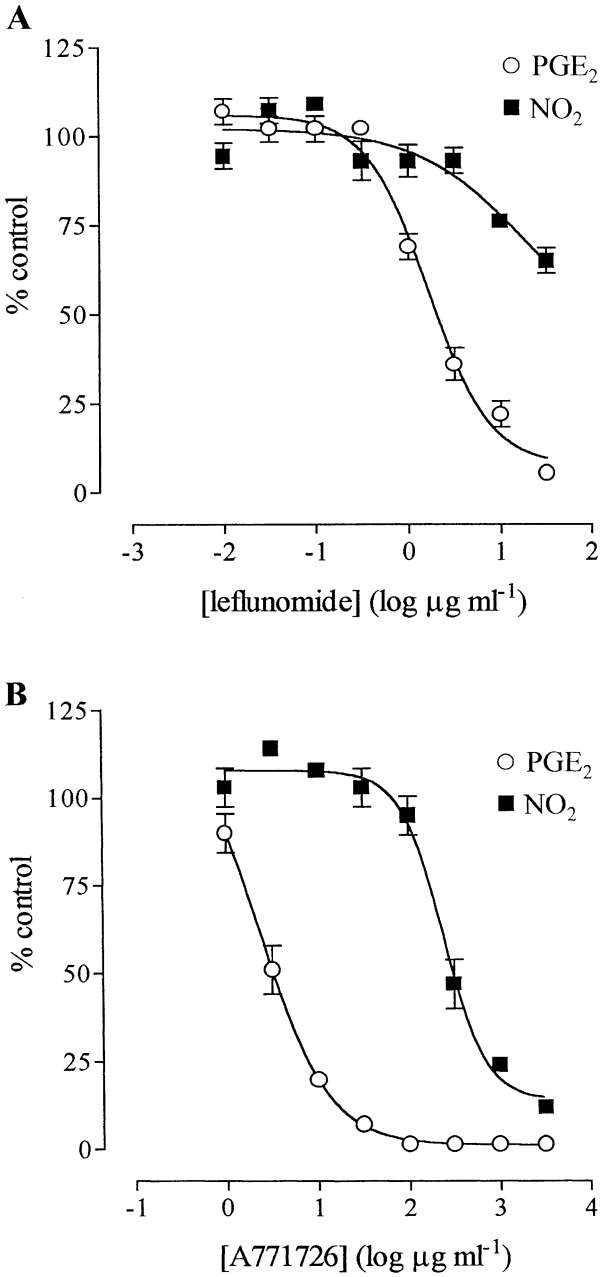

Figure 1 Inhibition by (A) leflunomide and (B) A771726 of the accumulation of PGE_2 and NO_2 in the culture medium bathing J774 macrophages exposed to LPS for 24 h ($n = 9-12$ for each point).

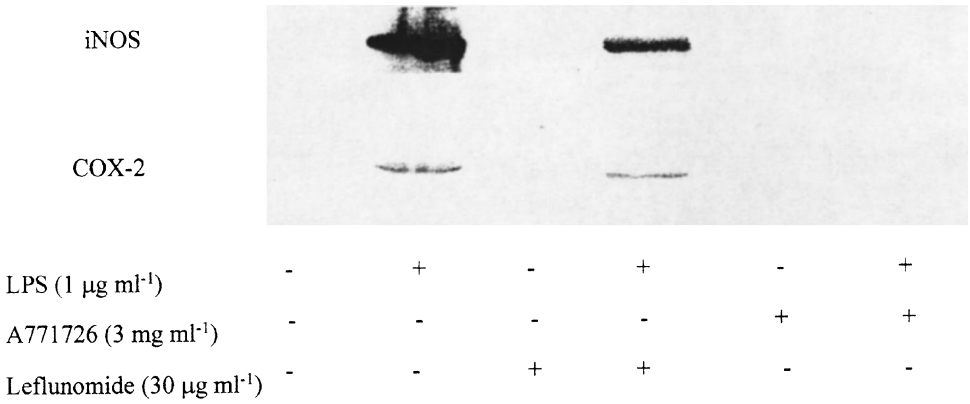

Figure 2 Expression of iNOS (upper panel) and COX-2 (lower panel) proteins in J774 macrophages as detected by Western blot analysis. Cells were treated with LPS plus leflunomide, A771726, or vehicle for 24 h.

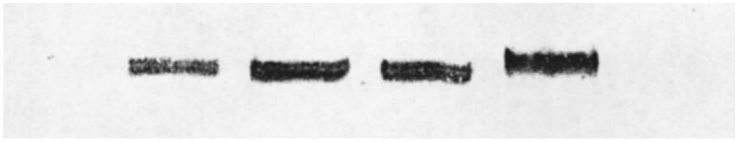

Figure 3 Expression of COX-2 protein in J774 macrophages as detected by Western blot analysis. Cells were treated wtih LPS plus either vehicle or increasing concentrations of A771726 for 24 h.

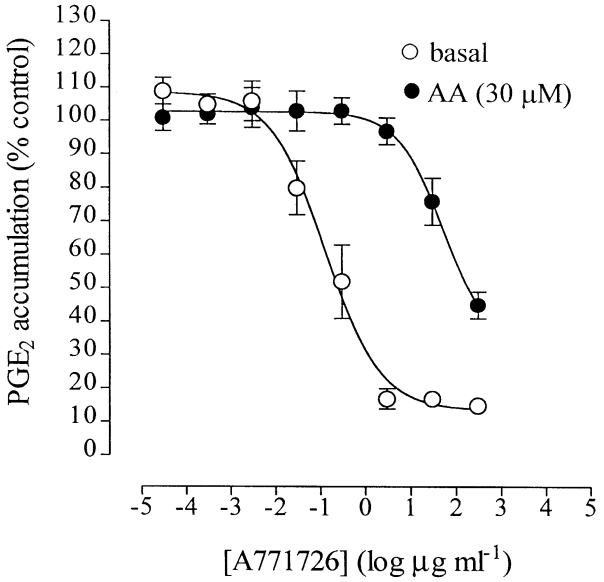

Figure 4 Inhibition by A771726 of the accumulation of PGE_2 in culture medium bathing A549 cells exposed to LPS for 24 h ($n=16-18$) either basally or following incubation with arachidonic acid (AA, 30 μM) for 30 min ($n=16-18$).

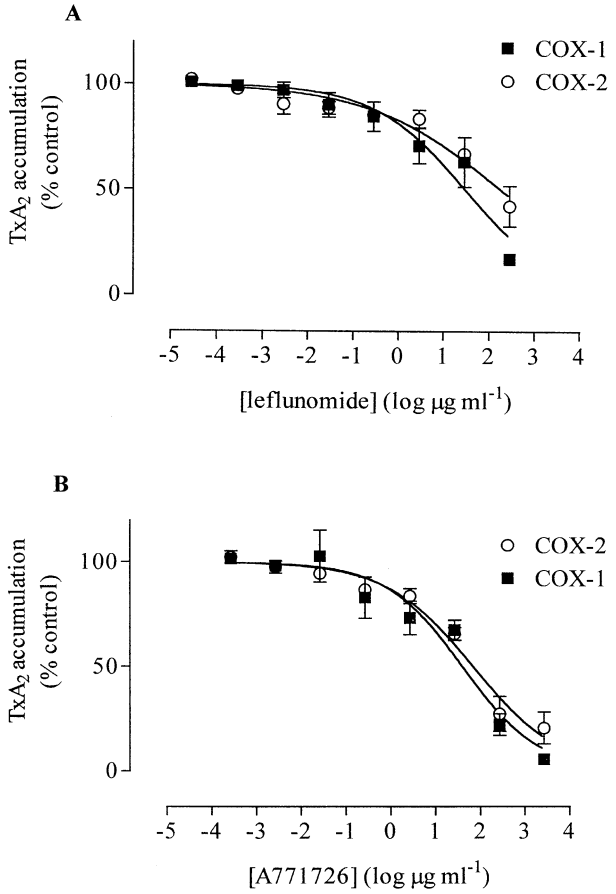

Figure 5 Inhibition by (A) leflunomide and (B) A771726 of the activities of COX-1 and COX-2 in human whole blood determined as the accumulation of TxB_2. ($n=5$).

2). Leflunomide at the maximum dissolvable concentration (30 $\mu g\ ml^{-1}$) was without effect on the expression of either COX-2 or iNOS (Figure 2). A771726 significantly inhibited the expression of both COX-2 and iNOS but only at concentrations greater than 300 $\mu g\ ml^{-1}$ (Figures 2 and 3).

Stimulation of COX-2 activity in A549 cells: effect of exogenous arachidonic acid on A771726 potency

Treatment of A549 cells with IL-1β for 24 h increased the accumulation of PGE_2 (0.04 ± 0.0 ng ml^{-1} to 5.83 ± 0.44 ng ml^{-1}, $n=12$). This increase was unaffected by leflunomide (up to 30 $\mu g\ ml^{-1}$, data not shown) but was inhibited in a concentration-dependent manner by A771726 (IC_{50} value of 0.13 $\mu g\ ml^{-1}$) (Figure 4).

Incubation of IL-1β-pre-treated A549 cells with exogenous arachidonic acid (30 μM) for 30 min caused the accumulation of 63 ± 5 ng ml^{-1} PGE_2 in the culture medium. Under these conditions of rapid PGE_2 formation, the activity of A771726 was reduced by approximately 400-fold (IC_{50} 52 $\mu g\ ml^{-1}$) (Figure 4).

Cell viability

At the highest doses used, neither leflunomide nor A771726 decreased cell viability either alone or in combination with LPS.

Human whole blood assay

In human whole blood the formations of TxB_2 in the COX-1 and COX-2 assays were, respectively, 42.08 ± 11.21 and 14.69 ± 2.37 ng ml^{-1} ($n=5$). These increases were weakly inhibited by both leflunomide (IC_{50} COX-1, 31 $\mu g\ ml^{-1}$; IC_{50} COX-2, 185 $\mu g\ ml^{-1}$; $n=5$) and A771726 (IC_{50} COX-1, 40 $\mu g\ ml^{-1}$; IC_{50} COX-2, 69 $\mu g\ ml^{-1}$; $n=5$) (Figure 5).

Inhibition of iNOS and COX-2 activity in vivo*: effects of leflunomide and A771726*

Infusion of LPS to rats caused a marked time-dependent increase in the plasma concentrations of NO_2/NO_3 ($t=0$ h, 9 ± 2.7 μM to $t=6$ h, 292 ± 83 μM; $n=8$). There was a smaller, less marked increase in the circulating levels of 6-keto-$PGF_{1\alpha}$ ($t=0$ h, 0.14 ± 0.10 ng ml^{-1} to $t=6$ h, 6.5 ± 2.48 ng ml^{-1};

$n=8$). These increases were associated with a general induction of iNOS and COX-2 throughout the vasculature as demonstrated by immunohistochemistry (data not shown).

Administration of a bolus of arachidonic acid (3 mg kg^{-1}, i.v.) to vehicle-treated rats at $t=6$ h caused, after 1 min, the plasma concentration of 6-keto-PGF$_{1\alpha}$ to rise to 2.75 ± 1.02 ng ml^{-1} ($n=8$). Infusion of rats with LPS for 6 h magnified this rise in 6-keto-PGF$_{1\alpha}$ to 404 ± 135 ng ml^{-1} (Figure 6A). The injection of arachidonic acid was without affect on the plasma levels of NO_2/NO_3 in any study groups (data not shown).

Leflunomide 20 mg kg^{-1}, inhibited the elevation in plasma 6-keto-PGF$_{1\alpha}$ caused by LPS infusion alone but was without effect against the rapid increase in plasma 6-keto-PGF$_{1\alpha}$ caused by injection of arachidonic acid in these animals. Leflunomide could not be applied at higher doses due to its poor solubility. However, experiments with leflunomide's active metabolite A771726 indicated that higher doses were required to inhibit the increase in 6-keto-PGF$_{1\alpha}$ following arachidonic acid injection than that following infusion of LPS alone (Figure 6B). A771726 (50 mg kg^{-1}) but not leflunomide (20 mg kg^{-1}) also reduced the rise in plasma 6-keto-PGF$_{1\alpha}$ levels following bolus injection of arachidonic acid in non-LPS-treated animals (Figure 6C).

Neither leflunomide nor A771726 inhibited the rise in plasma NO_2/NO_3 concentration caused by treatment of rats with LPS (Figure 7).

Cardiovascular parameters

LPS infusion (0.2 mg kg^{-1} h^{-1}) resulted in a time-dependent reduction in mean arterial blood pressure from 114 ± 7 mmHg to 90 ± 5 mmHg ($n=8$). Neither leflunomide nor A771726 affected the cardiovascular changes (Table 1).

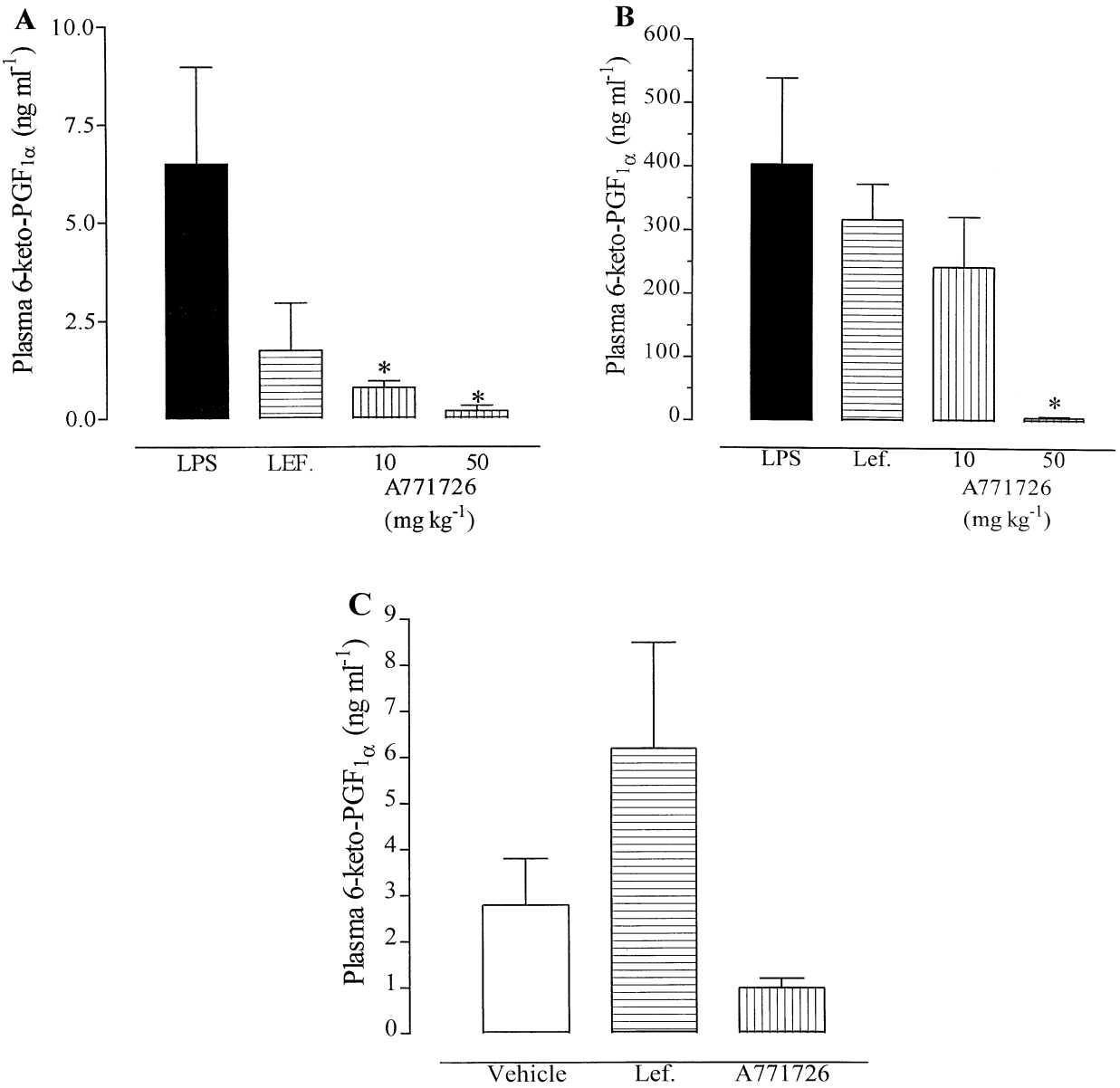

Figure 6 Effect of leflunomide (20 mg kg^{-1}) or A771726 (10 mg kg^{-1}) on the concentration of 6-keto-PGF$_{1\alpha}$ in the plasma of anaesthetized rats (A) treated with LPS (0.2 mg kg^{-1} h^{-1}) for 6 h. (B) Samples taken 1 min after injection of arachidonic acid (3 mg kg^{-1}, i.v.) to rats previously infused for 6 h with LPS. (C) Samples taken 1 min after injection of arachidonic acid (3 mg kg^{-1}, i.v.) to non-LPS treated rats. Results are expressed as mean$\pm$s.e.mean of 5–8 observations. *Denotes $P<0.05$, ANOVA + Bonferroni's test.

Discussion

Leflunomide, primarily used as an immunosuppressive drug, is also efficicacious in inflammatory models (Bartlett *et al*., 1993). Here we demonstrate, both *in vitro* and *in vivo* that leflunomide, probably *via* its active metabolite A771726, can directly inhibit the activity of cyclo-oxygenase. This effect could explain some of the anti-inflammatory activities of leflunomide.

To assess the action of leflunomide and A771726 on both the expression and activity of COX-2 and iNOS, J774.2 macrophages were incubated with LPS for 24 h, to induce the expression of these two enzymes (Swierkosz *et al*., 1995). Leflunomide and A771726 inhibited the accumulation of PGE_2 equi-potently. Notably, this inhibition was found at concentrations that did not affect the expression of COX-2. Indeed, more than 100 fold excess of A771726 was needed to produce such an effect. A771726 also inhibited the accumultion of NO_2 in the medium, but only at a high concentration consistent with inhibition of iNOS protein expression. The data from these experiments indicate that leflunomide, through the action of A771726, can inhibit prostanoid formation by inhibiting COX activity at low concentrations and COX expression at high concentrations. High concentrations of A771726 can similarly inhibit the production of NO_2 by inhibiting the expression of iNOS protein. This suggests that A771726 may inhibit some common pathway leading to the expression of COX-2 and iNOS in J774.2 macrophages. Potentially this could be by an influence on tyrosine kinase, for the induction of iNOS and COX-2 is inhibited by the tyrosine kinase inhibitors, erbstatin or herbimycin (Akarasereenont *et al*., 1994; Marczin *et al*., 1993). Furthermore, A771726 has been reported to inhibit Src tyrosine kinases (IC_{50} value, 40 μM) (Mattar *et al*., 1993). However, the IC_{50} value for this effect is far below that required in our experiments to inhibit the expression of COX-2 and iNOS. It would appear unlikely, therefore, that it is an influence on Src tyrosine kinases that explains the inhibitory effects of A771726 on the expression of iNOS and COX-2.

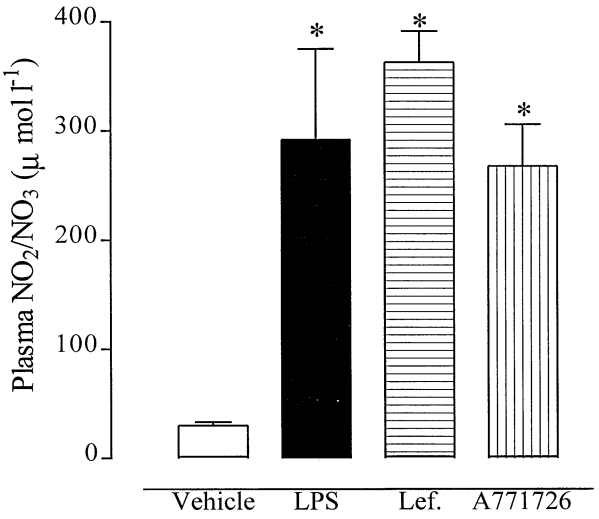

Figure 7 Effect of leflunomide (20 mg kg^{-1}) or A771726 (50 mg kg^{-1}) on the concentration of NO_2/NO_3 in the plasma of anaesthetized rats treated with LPS (0.2 mg kg^{-1} $^{-1}$) for 6 h. Results are expressed as mean $\pm$ s.e.mean of 5–8 observations. *$P<0.05$ ANOVA plus Dunnett's test.

The potencies of NSAIDs as inhibitors of COX can be affected by the supply of arachidonic acid, as has previously been demonstrated in A549 cells (Mitchell *et al*., 1994; 1997). We found that comparing the potencies of A771726 as an inhibitor of the formation of PGE_2 basally compared to the PGE_2 formation following incubation with 30 μM arachidonic acid revealed a 400 fold shift in IC_{50} value. This clearly indicates that A771726 can act as a weak, direct inhibitor of COX that is overcome by the presence of high concentrations of endogenous substrate, as has been found for sodium salicylate (Mitchell *et al*., 1997). Similarly, when A771726 was tested in isolated purified ovine COX-1 and COX-2 preparations (Curnock *et al*., 1997) in the presence of 10 μM exogenous arachidonic acid high IC_{50} values were obtained. Such results have been taken as evidence that A771726 is not a COX inhibitor. However, estimates of drug potency are more accurate in assays where endogenous arachidonic acid is used (Laneuville, 1994). We can therefore, conclude, that A771726 is a direct, though weak, inhibitor of COX activity *in vitro* (Curnock *et al*., 1997).

The potencies of NSAIDs can also be affected by binding to plasma proteins. This may well explain the relatively weak effects of both leflunomide and A771726 that we found in the human whole blood assay. Interestingly, the two compounds were found to be approximately equipotent against both COX-1 and COX-2. This could be explained by a conversion of leflunomide to A771726 within the whole blood, or by the two compounds acting in similar weakly competitive manners to inhibit both COX-1 and COX-2 in this assay system. With regard to the use of leflunomide in humans, our data would suggest that at achievable plasma concentrations, bearing in mind that leflunomide may be used at doses of approximately 20–100 mg (Smolen *et al*., 1999), neither leflunomide nor A771726 would have much direct effect on the activities of COX-1 or COX-2.

In vivo, A771726 (10 mg kg^{-1} or 50 mg kg^{-1}), but not leflunomide (20 mg kg^{-1}), inhibited the increase in circulating plasma 6-keto-$PGF_{1\alpha}$ induced by infusion of LPS (Figure 5). This is consistent with data obtained from a rat air pouch model in which leflunomide (25 mg kg^{-1}) was found to cause only a small (23%) reduction in PGE_2 accumulation (Curnock *et al*., 1997). Due to its poor solubility, leflunomide could not be administered at a higher concentration, but A771726 at the top dose used did significantly inhibit 6-keto-$PGF_{1\alpha}$ accumula-

Table 1 Mean arterial blood pressures of anaesthetized rats (mmHg) after 0, 2, 4 and 6 h of infusion

Treatment	n	*0*	*2*	*4*	*6*
Control	8	97±7	92±5	97±4	91±5
LPS + Vehicle (saline)	8	114±7	94±6	96±5	90±5*
LPS + Leflunomide (20 mg kg^{-1})	7	101±6	96±5	94±4	86±5*
LPS + A771726 (10 mg kg^{-1})	6	102±5	80±8	83±10	78±9*
LPS + A771726 (50 mg kg^{-1})	5	92±4	91±7	89±8	71±7*

*Denotes significant difference ($P<0.05$, unpaired t-test) between $t=0$ h and $t=6$ h within individual groups.

tion. This data is consistent with our hypothesis that the potency of COX inhibitors *in vivo* can be affected by the supply of arachidonic acid (Hamilton *et al*., 1999). Furthermore, the *in vivo* data correlate with the *in vitro* and whole blood experiments that demonstrate A771726 to be a weak, direct inhibitor of COX activity. A771726 also inhibited the formation of 6-keto-$PGF_{1\alpha}$ following the bolus injection of arachidonic acid. However, as in the A549 cells, this effect was only seen at the highest dose used. A771726 and leflunomide also failed to affect the accumulation of NO_2/NO_3 in the plasma, even at the highest doses used. This result is unsurprising, as in our *in vitro* experiments we only found an effect of A771726 on NO_2 production and iNOS expression at concentrations greater than 300 μg ml^{-1}. This concentration would be in excess of those achievable in our *in vivo* studies.

LPS infusion induced a time-dependent fall in mean arterial blood pressure that was not prevented by leflunomide or A771726 (Table 1). It has been previously proposed that this LPS-induced fall is largely related to the induction of iNOS and the overproduction of NO (Thiemerman, 1993; Szabo *et al*., 1994). Indeed, in previous experiments using this model, we found the selective iNOS inhibitor 1400W prevented the fall in blood pressure caused by LPS (Hamilton *et al*., 1998). As neither A771726 (10 and 50 mg kg^{-1}) nor leflunomide affected the production of NO_2/NO_3 *in vivo* it is unsurprising that they also had no effect on the blood pressure.

In conclusion, leflunomide either directly, or through its active metabolite A771726, inhibits the accumulation of prostaglandin at concentrations lower than those required to inhibit the induction of COX-2 protein. Direct inhibition of COX activity can therefore contribute to leflunomide's anti-inflammatory activity. High levels of arachidonic acid and possibly plasma binding appear to greatly limit this activity, supporting the idea that in inflammatory states leflunomide exerts its anti-inflammatory effects through other mechanisms. It does, however, appear unlikely that these other mechanisms could include inhibition of the expression of either COX-2 or iNOS.

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References

AKARASEREENONT, P., MITCHELL, J.A., APPLETON, I., THIEMERMANN, C. & VANE, J.R. (1994). Involvement of tyrosine kinase in the induction of cyclo-oxygenase and nitric oxide synthase by endotoxin in cultured cells. *Br. J. Pharmacol.,* **113,** 1522 – 1528.

BARTLETT, R.R., ANAGNOSTOPULOS, H., ZIELINSKI, T., MATTAR, T. & SCHLEYERBACH, R. (1993). Effects of leflunomide on immune responses and models of inflammation. *Springer Semin. Immunopathol.,* **14,** 381 – 394.

BARTLETT, R.R., DIMITRIJEVIC, M., MATTAR,A T., ZIELINSKI, T., GERMANN, T., RUDE, E., THOENES, G.H., KUCHLE, C.C., SCHORLEMMER H.U. & BREMER, E. (1991). Leflunomide (HWA 486), a novel immunomodulating compound for the treatment of autoimmune disorders and reactions leading to transplant rejection. *Agents Actions,* **32,** 10 – 21.

CURNOCK, A.P., ROBSON, P.A., YEA, C.M., MOSS, D., GADHER, S., THOMSON, T.A., WESTWOOD, R., RUUTH, E. & WILLIAMSON, R.A. (1997). Potencies of leflunomide and HR325 as inhibitors of prostaglandin endoperoxide H synthase-1 and -2: comparison with nonsteroidal anti-inflammatory drugs. *J. Pharmacol. Exp. Ther.,* **282,** 339 – 347.

GIERSE, J.K., KOBOLDT, C.M., WALKER, M.C., SEIBERT, K. & ISAKSON, P.C. (1999). Kinetic basis for selective inhibition of cyclooxygenases. *Biochem. J.,* in press.

HAMILTON, L.C. & WARNER, T.D. (1998). Interactions between inducible isoforms of nitric oxide synthase and cyclo-oxygenase *in vivo*: investigations using the selective inhibitors 1400W and celecoxib. *Br. J. Pharmacol.,* **125,** 330 – 340.

HAMILTON, L.C., MITCHELL, J.A., TOMLINSON, A. & WARNER, T.D. (1999). Synergy between cyclooxygenase-2 induction and arachidonic acid supply *in vivo*: consequences for nonsteroidal anti-inflammatory drug efficacy. *FASEB J.,* **13,** 245 – 251.

HAMILTON, L.C., VOJNOVIC, I., BAKHLE, Y.S., WARNER, T.D. & VANE, J.R. (1997). The anti-inflammatory drug leflunomide inhibits *in vitro* and *in vivo* the activity of COX-2 more potently than the induction of COX-2 or iNOS. *Br. J. Pharmacol.,* **120,** 49.

HLA, T., RISTIMAKI, A., APPLEBY, S. & BARRIOCANAL, J.G. (1993). Cyclooxygenase gene expression in inflammation and angiogenesis. *Ann. N.Y. Acad. Sci.,* **696,** 197 – 204.

LANEUVILLE, O., BREUER, D.K., DEWITT, D.L., HLA, T., FUNK, C.D. & SMITH, W.L. (1994). Differential inhibition of human prostaglandin endoperoxide H synthases-1 and -2 by nonsteroidal anti-inflammatory drugs. *J. Pharmacol. Exp. Ther.,* **271,** 927 – 934.

LORSBACH, R.B., MURPHY, W.J., LOWENSTEIN, C.J., SNYDER, S.H. & RUSSELL, S.W. (1993). Expression of the nitric oxide synthase gene in mouse macrophages activated for tumor cell killing. Molecular basis for the synergy between interferon-gamma and lipopolysaccharide. *J. Biol. Chem.,* **268,** 1908 – 1913.

MARCZIN, N., PAPAPETROPOULOS, A. & CATRAVAS, J.D. (1993). Tyrosine kinase inhibitors suppress endotoxin and IL-1 beta induced NO synthesis in aortic smooth muscle cells. *Am. J. Physiol.,* **265,** H1014 – H1018.

MATTAR, T., KOCHHAR, K., BARTLETT, R., BREMER, E.G. & FINNEGAN, A. (1993). Inhibition of the epidermal growth factor receptor tyrosine kinase activity by leflunomide. *FEBS Lett.,* **334,** 161 – 164.

MITCHELL, J.A., AKARASEREENONT, P., THIEMERMANN, C., FLOWER, R.J. & VANE, J.R. (1993). Selectivity of nonsteroid anti-inflammatory drugs as inhibitors of constitutive and inducible cyclo-oxygenase. *Proc. Natl. Acad. Sci. U.S.A.,* **90,** 11693 – 11697.

MITCHELL, J.A., BELVISI, M.G., AKARASEREENONT, P., ROBBINS, R.A., KWON, O.J., CROXTALL, J., BARNES, P.J. & VANE, J.R. (1994). Induction of cyclo-oxygenase-2 by cytokines in human pulmonary epithelial cells: regulation by dexamethasone. *Br. J. Pharmacol.,* **113,** 1008 – 1014.

MITCHELL, J.A., SAUNDERS, M., BARNES, P.J., NEWTON, R. & BELVISI, M.G. (1997). Sodium salicylate inhibits cyclo-oxygenase-2 activity independently of transcription factor (nuclear factor kappaB) activation: role of arachidonic acid. *Mol. Pharmacol.,* **51,** 907 – 912.

MLADENOVIC, V., DOMLJAN, Z., ROZMAN, B., JAJIC, I., MITHAJLOVIC, D., DORDEVIC, J., POPOVIC, M., DIMITRIJEVIC, M., ZIKOVIC, M., CAMPION, G., MUSIKIC, P., LOWFRIEDRICH, I., OED, C., SEIFERT, H. & STRAND, V. (1995). Safety and effectiveness of leflunomide in the treatment of patients with active rheumatoid arthritis. *Arthritis Rheum.,* **38,** 1595 – 1603.

SAUNDERS, M.A., BELVISI, M.G., CORDEN, M.B., FOX, A.J., EVANS, T.W., BARNES, P.J. & MITCHELL, J.A. (1996). Exacerbation of the release of prostaglandin (PG) E2 by bradykinin after COX-2 induction in human airway epithelial cells. *Br. J. Pharmacol.,* **119,** P46.

SEIBERT, K. & MASFERRER, J.L. (1994). Role of inducible cyclooxygenase (COX-2) in inflammation. *Receptor,* **4,** 17 – 23.

SILVA, J.H. & MORRIS, R.E. (1997). Leflunomide and malononitrilamides. *Am. J. Med. Sci.,* **313,** 289 – 301.

SMOLEN, J.S., KALDEN, J.R., SCOTT, D.L., ROZMAN, B., KVIEN, T.K., LARSEN, A., LOEW-FRIEDRICH, I., OLD, C. & ROSENBURG, R. (1999). Efficacy and safety of leflunomide compared with placebo and sulphasalazine in active rheumatoid arthritis: a double-blind, randomised, multicentre trial. European Leflunomide Study Group. *Lancet,* **353,** 259–266.

STEUR, D.J. & MARLETTA, M.A. (1989). Induction of nitrite/nitrate synthesis in murine macrophages by BCG infection, lymphokines or interferon-gamma. *J. Immunol.,* **139,** 518–525.

SWIERKOSZ, T.A., MITCHELL, J.A., WARNER, T.D., BOTTING, R.M. & VANE, J.R. (1995). Co-induction of nitric oxide synthase and cyclo-oxygenase: interactions between nitric oxide and prostanoids. *Br. J. Pharmacol.,* **114,** 1335–1342.

SZABO, C., SOUTHAN, G.J. & THIEMERMANN, C. (1994). Beneficial effects and improved survival in rodent models of septic shock with S-methylisothiourea sulfate, a potent and selective inhibitor of inducible nitric oxide synthase. *Proc. Natl. Acad. Sci. U.S.A.,* **91,** 12472–12476.

THIEMERMANN, C. (1993). Drugs which modulate nitric oxide homeostasis are likely to provide therapeutic benefit in cardiovascular disease. *Cardiovasc. Res.,* **27,** 2104.

TOMLINSON, A., APPLETON, I., MOORE, A.R., GILROY, D.W., WILLIS, D., MITCHELL, J.A. & WILLOUGHBY, D.A. (1994). Cyclo-oxygenase and nitric oxide synthase isoforms in rat carrageenin-induced pleurisy. *Br. J. Pharmacol.,* **113,** 693–698.

VANE, J.R.. (1971). Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nature New Biol.,* **231,** 232–235.

VANE, J.R., MITCHELL, J.A., APPLETON, I., TOMLINSON, A., BISHOP-BAILEY, D., CROXTALL, J. & WILLOUGHBY, D.A. (1994). Inducible isoforms of cyclooxygenase and nitric-oxide synthase in inflammation. *Proc. Natl. Acad. Sci. U.S.A.,* **91,** 2046–2050.

XIE, Q.W., CHO, H.J., CALAYCAY, J., MUMFORD, R.A., SWIDEREK, K.M., LEE, T.D., DING, A., TROSO, T. & NATHAN, C. (1992). Cloning and characterisation of inducible nitric oxide synthase from murine macrophages. *Science,* **256,** 225–228.

XIE, W.L., CHIPMAN, J.G., ROBERTSON, R.L., ERIKSON, R.L. & SIMMONS, D.L. (1991). Expression of a mitogen responsive gene encoding prostaglandin synthase is regulated by mRNA splicing. *Proc. Natl. Acad. Sci. U.S.A.,* **88,** 2692–2696.

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