

**INHIBITION OF INDUCIBLE NITRIC OXIDE SYNTHASE EXPRESSION BY
NOVEL NONSTEROIDAL ANTI-INFLAMMATORY DERIVATIVES WITH
GASTROINTESTINAL-SPARING PROPERTIES.**

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Running Title: Inhibition of nitric oxide synthase expression

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Summary

1. Effects of novel nitric oxide-releasing nonsteroidal anti-inflammatory compounds (NO-NSAIDs) on induction of nitric oxide (NO) synthase by bacterial lipopolysaccharide (LPS) were examined in a cultured murine macrophage cell line J774.

2. LPS-induced nitrite production was markedly attenuated by the nitroxybutylester derivatives of flurbiprofen (FNBE), aspirin, ketoprofen, naproxen, diclofenac and ketorolac, with each compound reducing accumulated nitrite levels by >40% at the maximum concentrations (100 $\mu\text{g ml}^{-1}$) used.

3. Further examination revealed that nitrite production was inhibited in a concentration-dependent (1-100 $\mu\text{g ml}^{-1}$) manner by FNBE which at 100 $\mu\text{g ml}^{-1}$ decreased LPS-stimulated levels by $63.3 \pm 8.6\%$ (n=7). The parent compound flurbiprofen was relatively ineffective over the same concentration range, inhibiting nitrite accumulation by $24 \pm 0.9\%$ (n=3) at the maximum concentration used (100 $\mu\text{g ml}^{-1}$).

4. FNBE reduced LPS-induced nitrite production when added to cells up to 4 h after LPS. Thereafter, FNBE caused very little or no reduction in nitrite levels. Furthermore NO-NSAIDs (100 $\mu\text{g ml}^{-1}$) did not inhibit the metabolism of L-[^3H]arginine to citrulline by NO synthase isolated from LPS-activated macrophages.

5. Western blot analysis demonstrated that NO synthase expression was markedly attenuated following co-incubation of J774 cell with LPS (1 $\mu\text{g ml}^{-1}$; 24 h) and FNBE (100 $\mu\text{g/ml}$; 24 h). Thus taken together, these findings indicate that NO-NSAIDs inhibit induction of NO synthase

without directly affecting enzyme activity.

6. In conclusion our results indicate that NO-NSAIDs can inhibit the inducible L-arginine-NO pathway, and are capable of suppressing NO synthesis by inhibiting expression of NO synthase. The clinical implications of these findings remain to be established.

Key Words: Macrophage; Inflammation; Nitric oxide; Bacterial lipopolysaccharide; Nonsteroidal anti-inflammatory drugs; Inducible nitric oxide synthase.

INTRODUCTION

Nonsteroidal anti-inflammatory compounds (NSAIDs) are amongst the most widely used drugs, proving beneficial in preventing several of the deleterious effects associated with the pathophysiology of inflammation. The application of these agents is however generally limited by their significant untoward effects on the gastrointestinal tract, due mainly to suppression of prostaglandin synthesis and the subsequent reduction in vascular perfusion within the gastric mucosa (Roth, 1988; Allison *et al.*, 1992, Wallace & Granger, 1992).

In view of the fact that nitric oxide (NO) increases blood flow to the gastric mucosa (MacNaughton *et al.*, 1989; Lippe & Holzer, 1992; Tepperman & Whittle, 1992) we proposed that incorporating a NO-releasing moiety into conventional NSAIDs may attenuate their cytotoxic effects. This hypothesis has now been tested using nitroxybutylester derivatives (NO-NSAIDs) of several orthodox NSAIDs including flurbiprofen and aspirin. When applied *in vivo* some of these novel compounds have been shown to retain the potent anti-inflammatory properties of the parent drug but more importantly they possess markedly reduced ulcerogenic effects (Wallace *et al.*, 1994a; 1994b; 1994c). This gastrointestinal-sparing effect of the NO-NSAIDs is presumably due to the liberation of NO following metabolism. In this regard we have, in preliminary studies, demonstrated that flurbiprofen-nitroxybutylester (FNBE) raises cGMP levels in human cultured umbilical vein endothelial cells (Baydoun *et al.*, 1995) and when applied *in vivo* elevates plasma nitrite levels in rats (Wallace *et al.*, 1994b).

Since both exogenous and endogenous NO have been shown to inhibit induction of NO synthase (Park *et al.*, 1994; Mariotto *et al.*, 1995a) and to block the expression of inducible cyclooxygenase (Swierkosz *et al.*, 1995) we have investigated whether selected NO-NSAIDs regulate induction of NO synthase and thus NO synthesis in LPS-activated J774 macrophages. A preliminary account of this work has been presented in abstract form (Cirino *et al.*, 1995).

METHODS

Cell culture

The murine macrophage cell line J774 was maintained in continuous culture in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4.4% NaHCO₃, penicillin (100 units ml⁻¹) and streptomycin (100 µg ml⁻¹), 2 mM glutamine and 10% foetal calf serum. Monolayers of cells were routinely harvested by gentle scraping with a Teflon cell-scraper, diluted 1:10 in fresh medium and cultured to confluency at 37°C.

Analysis of nitrite production by LPS-activated J774 cells

Prior to each experiment, cells were harvested by gentle scraping and plated at a seeding density of 10⁵ cells per well in 96-well microtiter plates. Confluent monolayers of cells were incubated for 24 h with either DMEM or DMEM containing *Escherichia Coli* lipopolysaccharide (LPS; 1 µg ml⁻¹) alone or in combination with nitroxybutylester derivatives (100 µg ml⁻¹) of flurbiprofen (FNBE), aspirin, ketoprofen, naproxen, diclofenac and ketorolac. FNBE was selected as the representative compound from this group and investigated further by examining its concentration-dependent (1-100 µg ml⁻¹) effect on nitrite production. The parent compound flurbiprofen (1-100 µg ml⁻¹) was also investigated. In another series of experiments, FNBE (100 µg ml⁻¹) was added to wells at various time points after exposure of cells to LPS (1 µg ml⁻¹). To determine whether FNBE (100 µg ml⁻¹) itself generated detectable quantities of nitrite, cells were incubated with this compound in the absence of LPS. Nitrite production was assayed after 24 h by measuring the accumulation levels in the culture medium using the Griess reaction (Green *et al.*, 1982), as described previously (Baydoun *et al.*, 1993).

Western blot analysis

Confluent monolayers of J774 cells in 24-well culture plates (5×10^5 cells per well) were incubated for 24 h with either fresh DMEM or DMEM containing LPS ($1 \mu\text{g ml}^{-1}$) alone or in combination with FNBE ($100 \mu\text{g ml}^{-1}$), dexamethasone ($1 \mu\text{M}$) or cycloheximide ($0.5 \mu\text{M}$). Incubations were terminated by rapid aspiration of the cell supernatant followed by washing with ice-cold phosphate buffered saline (7.3 g l^{-1} NaCl, 0.0027 g l^{-1} KCl, 0.02 g l^{-1} Na_2HPO_4 , 0.0015 g l^{-1} KHPO_4 , pH 7.4) containing $200 \mu\text{M Na}^+$ orthovanadate. Cells were lysed in buffer containing 63.5 mM Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, 5% β -mercaptoethanol, 1 mM Na^+ orthovanadate, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF) and $50 \mu\text{g ml}^{-1}$ leupeptin. Lysates ($20 \mu\text{g}$ protein per lane) were separated by SDS-PAGE on 8% polyacrylamide gel and transferred for 3 h at 0.8 mA cm^2 onto $0.2 \mu\text{m}$ nitrocellulose membrane (Anderman and Co., Kingston-upon-Thames, Surrey, U.K.). Membranes were blocked for 2 hours in 100 mM NaCl , 10 mM Tris , 0.1% (v/v) Tween-20, pH 7.4 (STT) containing 1% (w/v) BSA and subsequently probed overnight with mouse monoclonal anti-iNOS antibody (1/500 dilution in STT containing 0.2% (w/v) BSA). Blots were washed with STT ($6 \times 10 \text{ min}$) and incubated with horseradish peroxidase-conjugated rabbit anti-mouse IgG (1/10,000) for 1 hour. Following further washing ($8 \times 10 \text{ min}$) in STT, immunoreactive bands were visualised using ECL detection System (Amersham). Protein bands were quantified by scanning densitometry (UVP E.A.S.Y. Plus Enhanced Analysis System; Ultra-Violet Products Ltd, Cambridge, UK) and the data expressed as a percentage of the value obtained for samples from cells treated with LPS alone.

Citrulline assay

J774 macrophages (9.5×10^6 cells) in 75 cm² culture flasks were activated with LPS ($1 \mu\text{g ml}^{-1}$) for 24 h and harvested with a cell scraper into 1 ml of homogenization buffer containing 50 mM Tris-HCl, 320 mM sucrose, 1 mM EDTA, 1 mM dithiothreitol, $10 \mu\text{g ml}^{-1}$ leupeptin, $10 \mu\text{g ml}^{-1}$ soybean trypsin inhibitor and $2 \mu\text{g ml}^{-1}$ aprotinin. Cells were lysed by freezing the suspension at -70°C and thawing at 37°C three times. The lysate was then centrifuged at 10,000 g for 60 min at 4°C . Conversion of L-[³H]arginine to L-[³H]citrulline was determined as described by Brown *et al.* (1992). Briefly, 20 μl of 10,000 g supernatant was added to 100 μl of assay buffer consisting of 50 mM KH₂PO₄, 1 mM MgCl₂, 0.2 mM CaCl₂, 50 mM valine, 1 mM dithiothreitol, 100 μM NADPH, 1 mM L-citrulline, 20 μM L-arginine and $0.5 \mu\text{Ci ml}^{-1}$ L-[³H]arginine containing 2.7×10^{-11} moles L-arginine μCi^{-1} . When applied, inhibitors and other compounds were dissolved directly in the assay buffer. The reactions were monitored for 20 min at 37°C and L-[³H]citrulline separated from arginine by adding 1 ml of a 1:1 suspension of Dowex (AG 50W-8) in water to each sample. The supernatant was transferred into β -vials and radioactivity measured by liquid scintillation counting.

Cell Viability

Cell viability under different experimental conditions was determined by assessing mitochondrial-dependent reduction of [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) to formazan as described by Mosmann (1983). Briefly, after each experimental protocol, cells in 96-well plates were incubated with MTT (0.5 mg ml^{-1}) for 4 h at 37°C . An equal volume of 10% SDS in 0.01 M HCl was then added to all wells and incubated for a further 3 h to dissolve the accumulated crystals of formazan. Absorbance was read at 560 nm using a

Multiskan II plate reader (Titertek).

Materials

NO-NSAIDs were synthesised by NICOX Ltd (London, UK.). Tissue culture reagents were purchased from Gibco (Paisley, U.K.). LPS from *Escherichia coli* (serotype 0111:B4), flurbiprofen, cycloheximide, dexamethasone, MTT, N^G-nitro-L-arginine methylester, ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA) and Dowex (AG 50W-8) were obtained from Sigma (Poole, U.K.). Monoclonal antibody for inducible nitric oxide synthase was from Affiniti Research Products Ltd (Nottingham, U.K.). Other chemicals were from Sigma or BDH and of the highest analytical grade obtainable.

Statistics

All values are means \pm s.e. mean of measurements in at least three different cell cultures with 5 replicates per experiment. Statistical analyses were performed using a multiple means comparison test (Harper, 1984) validated by comparison with the Newman-Keuls multiple range test in the statistical package SPP (Royston, 1984) with the overall confidence levels set at 99% (0.01).

RESULTS

Inhibition of nitrite production by NO-NSAIDs

Incubation of J774 cells with LPS ($1 \mu\text{g ml}^{-1}$) stimulated nitric oxide production, elevating nitrite levels from a basal value of 0.02 ± 0.003 to 0.75 ± 0.01 nmoles $\mu\text{g protein}^{-1} 24 \text{ h}^{-1}$ ($n=13$). This increase in nitrite production was markedly attenuated by all the NO-NSAIDs which at $100 \mu\text{g ml}^{-1}$, decreased nitrite levels by greater than 40% (Fig. 1). Further studies revealed that FNBE reduced LPS-induced nitrite production in a concentration-dependent ($1-100 \mu\text{g ml}^{-1}$) manner, with $100 \mu\text{g ml}^{-1}$ decreasing accumulated nitrite levels by $63.3 \pm 8.6\%$ ($n=7$; Fig 2). In contrast, the parent compound flurbiprofen had no significant effect at lower concentrations ($1-30 \mu\text{g ml}^{-1}$) but reduced nitrite production marginally ($24.0 \pm 0.9\%$ inhibition) at the maximum concentration ($100 \mu\text{g ml}^{-1}$) used (Fig 2; $n=3$). None of these compounds significantly affected mitochondrial reduction of MTT to formazan (data not shown) suggesting that these NO-NSAIDs are not cytotoxic to J774 cells even at the highest concentration used.

Time course analysis revealed that the inhibition caused by FNBE was critically dependent on the time of addition of this compound. As shown in Fig. 3, FNBE significantly reduced LPS-induced NO production when added up to 4 h after exposure of J774 cells to LPS. Thereafter, FNBE had very little or no effect on nitrite production. Furthermore when incubated with cells for 24 h in the absence of LPS, FNBE ($100 \mu\text{g ml}^{-1}$) failed to elevate accumulated nitrite levels (0.027 ± 0.003 nmoles $\mu\text{g protein}^{-1} 24 \text{ h}^{-1}$; $n=3$) above basal values (0.025 ± 0.002 nmoles $\mu\text{g protein}^{-1} 24 \text{ h}^{-1}$; $n=3$), suggesting that nitrite released from this compound does not contribute to the total amount of detectable nitrite produced by LPS activated J774 macrophages.

Effects of NO-NSAIDs on iNOS activity

Unstimulated J774 cells had no detectable levels of NOS activity. Following activation

with LPS ($1 \mu\text{g ml}^{-1}$) total NOS activity was significantly elevated (36.7 ± 4.7 pmoles L- $[\text{}^3\text{H}]$ citrulline $\mu\text{g protein}^{-1} 20 \text{ min}^{-1}$; $n=3$) and was unaffected by removal of calcium from the reaction buffer using 1 mM EGTA. Conversion L- $[\text{}^3\text{H}]$ arginine to citrulline was, however, markedly attenuated by L-NAME, which at 100 μM inhibited L- $[\text{}^3\text{H}]$ citrulline production by $79.7 \pm 5.5\%$ ($n=3$). In contrast FNBE and all the other NO-NSAIDs failed to inhibit conversion of $[\text{}^3\text{H}]$ arginine to $[\text{}^3\text{H}]$ citrulline (Table 1).

Western blot analysis

To determine whether the inhibitory effect of FNBE was due to the inhibition of iNOS expression, Western blot analysis was carried out on whole cell lysates using a monoclonal antibody for murine iNOS. In lysates from LPS-activated ($1 \mu\text{g ml}^{-1}$; 24 h) but not from untreated cells the iNOS antibody recognised a protein band which migrated at a molecular weight of ~ 130 kDa (Fig 4a). The levels of iNOS protein, determined by scanning densitometry, were markedly attenuated in lysates from J774 cells activated with LPS in the presence of the protein synthesis inhibitor cycloheximide ($0.5 \mu\text{M}$), or the glucocorticoid dexamethasone ($1 \mu\text{M}$). Similarly, FNBE ($100 \mu\text{g ml}^{-1}$) reduced iNOS expression substantially and, although less effective than cycloheximide, was comparable to dexamethasone (Figs. 4a and b).

DISCUSSION

This study has examined the effects of novel nitric oxide-releasing nonsteroidal anti-inflammatory drugs on the inducible L-arginine-nitric oxide pathway using the murine macrophage cell line, J774. In these cells synthesis of NO from its precursor L-arginine is mediated by the Ca²⁺/calmodulin-insensitive NO synthase enzyme, which unlike its constitutive isoform (for review see Moncada *et al.*, 1991; Sessa, 1994) is only expressed following activation with LPS and/or other pro-inflammatory mediators (Hibbs *et al.*, 1987; Marletta *et al.*, 1988; Baydoun *et al.*, 1993). Under these conditions, induction of NO synthase is time-dependent, requires *de novo* protein synthesis and can be regulated by glucocorticoids such as dexamethasone (Di Rosa *et al.*, 1990; Assreuy & Moncada, 1992; Baydoun *et al.*, 1993) acting presumably at the level of gene transcription (Marumo *et al.*, 1993).

In this study we have identified NO-NSAIDs which inhibit this inducible NO generating pathway in J774 cells and our data are consistent with a short report by Mariotto *et al.* (1995b) showing that FNBE inhibits iNOS expression in neutrophils when administered to rats *in vivo*. These effects appear to be specific for the NO-NSAIDs since the conventional parent compound was relatively ineffective over the same concentration range. This is in contrast to the effects of NO-NSAIDs and NSAIDs on the cyclo-oxygenase (COX) pathway where both groups of compounds are virtually equipotent in inhibiting prostaglandin biosynthesis (Wallace *et al.*, 1994b, Mitchell *et al.*, 1994). Thus there may be differential regulation of NO synthase and COX enzymes by NO-NSAIDs.

With regard to NO synthase, our data demonstrate that the inhibition of nitrite production by the NO-NSAIDs is due to inhibition of enzyme expression rather than to a direct effect of FNBE on enzyme activity. Thus, co-incubation of J774 cells with LPS and FNBE resulted in a marked decrease in both accumulated nitrite levels and in NO synthase protein expression but

when added several hours after LPS, FNBE failed to inhibit nitrite production suggesting that once induced, the activity of NO synthase cannot be modified by FNBE. This was further confirmed by the fact that neither FNBE nor the other NO-NSAIDs examined inhibited conversion of L-[³H]arginine to citrulline by NO synthase isolated from LPS activated J774 cells. This latter finding is in contrast to those made by Assreuy *et al.* (1993) demonstrating that S-nitroso-acetyl-penicillamine and S-nitroso-glutathione, compounds capable of liberating NO, directly inhibited the activity of iNOS isolated from J774 cells. The reasons for these differences are as yet unclear but may be related to the amounts of NO produced by each compounds in solution. Unlike conventional NO donors, NO-NSAIDs do not generate substantial quantities of NO *in vitro* but release rather discrete amounts which are perhaps insufficient to directly inhibit iNOS activity once expressed. Indeed the quantities of NO produced by NO-NSAIDs appear to be in the low nanomolar range and below the limit of detection by the relatively insensitive Griess reaction. These levels are however detectable either directly by chemiluminescence (unpublished observations) or indirectly by monitoring changes in cGMP levels following incubation of NO-NSAIDs with cells (Baydoun *et al.*, 1995).

Although our data suggest that NO-NSAIDs, or at least FNBE, may be acting at the molecular level to inhibit NO synthase induction, the precise mechanism by which this effect is mediated still remains to be established. A recent report (Kopp & Ghosh, 1994) has suggested that aspirin and sodium salicylate inhibit the activation of nuclear factor kappa B (NF- κ B), a transcription factor activated by pro-inflammatory mediators (Collart *et al.*, 1990; Schreck *et al.*, 1992; Grilli *et al.*, 1993) and implicated in the transcription of a variety of genes involved in inflammation, including that for NO synthase (Mülsche *et al.*, 1993; Sherman *et al.*, 1993; Xie *et al.*, 1993; 1994). Whether NO-NSAIDs exert their effects by inhibiting activation of NF- κ B remains a critical experiment to be carried out. It is worth noting however that in the study carried

out by Kopp and Ghosh (1994), relatively high millimolar concentrations of aspirin or sodium salicylate were required to inhibit activation of NF- κ B.

In our study all the NO-NSAIDs examined significantly suppressed nitrite production (> 40%) at micromolar concentrations, which according to the findings of Kopp & Ghosh (1994) would be ineffective in blocking LPS-induced NF- κ B activation. Of course it is feasible that NO-NSAIDs are more potent inhibitors of NF- κ B, inhibiting activation of the latter either directly or indirectly via the release of NO. In this regard NO has recently been shown to cause inhibition of NF- κ B activation, exerting its effect by enhancing the expression and preventing the degradation of the cytoplasmic NF- κ B inhibitor, I κ B α (Peng *et al.*, 1995). This mechanism of action could account for the difference in potency between NO-releasing NSAIDs and conventional NSAIDs observed in our study. Furthermore it would be in agreement with a recent report demonstrating that sodium salicylate and aspirin inhibit NO synthase induction in rat alveolar macrophages when applied at millimolar but not micromolar concentrations (Aeberhard *et al.*, 1995).

The inhibition observed at higher concentrations by Aeberhard *et al.* (1995) was associated with a marked increase in lactate dehydrogenase release from cells. Since this is normally associated with membrane damage it therefore strongly suggests that the inhibition observed may simply be the result of the cytotoxic actions of the relatively high millimolar concentrations of NSAIDs used in their study. In our study the maximum concentration of drugs used was 100 $\mu\text{g ml}^{-1}$ corresponding to $\sim 300 \mu\text{M}$ for NO-NSAIDs or $\sim 500 \mu\text{M}$ for flurbiprofen. These concentrations were not cytotoxic since they did not modify mitochondrial activity assessed by monitoring mitochondrial reduction of MTT to formazan.

In conclusion our data have identified NO-NSAIDs as inhibitors of the inducible L-arginine-NO pathway in J774 macrophages, exerting their effects presumably by inhibiting the expression of iNOS. This novel finding provides an additional mechanism by which the anti-

inflammatory properties of these compounds could be mediated. Further studies determining plasma concentrations of these compounds are however required to clarify whether our observations have any clinical relevance particularly in chronic inflammation where the dominant inflammatory cells are macrophages and overproduction of NO at the sites of inflammation can augment the inflammatory response (Boughton-Smith *et al.*, 1993; McCartney-Francis *et al.*, 1993; Vane *et al.*, 1994; Weinberg *et al.*, 1994; Salvemini *et al.*, 1995).

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Figure Legends

Figure 1 Inhibition of LPS-induced nitrite production in J774 macrophages by NO-NSAIDs. Cells were incubated for 24 h with LPS ($1 \mu\text{g ml}^{-1}$) alone (control) and in combination with $100 \mu\text{g ml}^{-1}$ of nitroxybutylester derivatives of either flurbiprofen (FNBE), aspirin (ANBE), ketoprofen (KPNBE), naproxen (NNBE), diclofenac (DFNBE) or ketorolac (KRNBE). Accumulated nitrite in the culture medium was determined by the Griess reaction. Results are expressed as % of the LPS response (control; $0.72 \pm 0.01 \text{ nmoles nitrite } \mu\text{g protein}^{-1} 24 \text{ h}^{-1}$) and values are the mean \pm s.e. mean of at least 3 independent experiments with 5 replicates in each. All values are significantly different from control at $p < 0.01$.

Figure 2 Concentration-dependent inhibition of LPS-induced nitrite production in J774 macrophages by flurbiprofen-nitroxybutylester or flurbiprofen. Cells were incubated for 24 h with LPS ($1 \mu\text{g ml}^{-1}$) alone (control) and in combination with increasing concentrations (1 - $100 \mu\text{g ml}^{-1}$) of either FNBE (\bullet - \bullet) or flurbiprofen (\circ - \circ). Accumulated nitrite in the culture medium was determined by the Griess reaction. Results are expressed as % of the LPS response ($0.71 \pm 0.01 \text{ nmoles nitrite } \mu\text{g protein}^{-1} 24 \text{ h}^{-1}$) and values are the mean \pm s.e. mean of at least 3 independent experiments with 5 replicates in each. * denotes values significantly different from control at $P < 0.01$.

Figure 3 Time-dependent inhibition of LPS-induced nitrite production in J774 macrophages by

flurbiprofen-nitroxybutylester. Cells were incubated with LPS ($1 \mu\text{g ml}^{-1}$) alone (control) and in combination with FNBE ($100 \mu\text{g ml}^{-1}$) added at 0-12 h after LPS. Accumulated nitrite in the culture medium was determined 24 h after addition of LPS by the Griess reaction. Results are expressed as % of the LPS response ($0.74 \pm 0.016 \text{ nmoles nitrite } \mu\text{g protein}^{-1} 24 \text{ h}^{-1}$) and values are the mean \pm s.e. mean of at least 3 independent experiments with 5 replicates in each.

* denotes values significantly different from control at $P < 0.01$.

Figure 4 A: Western blot analysis of inducible NO synthase in J774 cells. Lysates ($20 \mu\text{g}$ protein) from untreated cells (C) and from cells incubated with LPS ($1 \mu\text{g ml}^{-1}$; 24 h) alone and in combination with either dexamethasone ($1 \mu\text{M}$; Dex), cycloheximide ($0.5 \mu\text{M}$; Cyc) or flurbiprofen-nitroxybutylester ($100 \mu\text{g ml}^{-1}$; FNBE) were separated by SDS-PAGE, transferred to a nitrocellulose and blotted with a mouse monoclonal anti-iNOS antibody. This blot is representative of 3 independent experiments. B: Relative intensity of iNOS protein bands quantitated by scanning densitometry. Data are expressed as a percentage of the maximum value obtained for cells treated with LPS alone.

Table 1 Effects of NO-NSAIDs on isolated NO synthase activity.

	L-[³ H]citrulline (pmoles $\mu\text{g protein}^{-1}$ 20 min ⁻¹)
Control	36.7 \pm 4.7
EGTA	30.2 \pm 2.9
L-NAME	7.4 \pm 2.1*
FNBE	36.5 \pm 2.7
ANBE	37.8 \pm 2.5
KPNBE	42.8 \pm 4.5
NNBE	36.3 \pm 2.0
DFNBE	39.2 \pm 2.7
KRNBE	38.6 \pm 3.7

J774 cells were activated with LPS (1 $\mu\text{g ml}^{-1}$; 24 h) and NO synthase isolated from freeze-thawed lysates by centrifugation. The 10,000 x g supernatant was assayed for enzyme activity in the absence (LPS Control) and presence of either EGTA (1 mM), N^G-nitro-L-arginine methyl ester (L-NAME; 100 μM) or nitroxybutylester derivatives (100 $\mu\text{g ml}^{-1}$) of flurbiprofen (FNBE), aspirin (ANBE), ketoprofen (KPNBE), naproxen (NNBE), diclofenac(DFNBE) or ketorolac (KRNBE). L-[³H]citrulline production is expressed as pmoles L-[³H]citrulline $\mu\text{g protein}^{-1}$ 20 min⁻¹). Values are the mean \pm s.e. mean of 3 independent experiments with 3