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Induction of cyclooxygenase and nitric oxide synthase in endotoxin-activated J774 macrophages is differentially regulated by indomethacin: Enhanced cyclooxygenase-2 protein expression but reduction of inducible nitric oxide synthase

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Abstract

The involvement of prostaglandins in feedback modulation of the lipopolysaccharide-inducible isoforms of cyclooxygenase and nitric oxide was studied. This was done by testing the effects of arachidonic acid and indomethacin in the murine macrophage cell line J774. When added before lipopolysaccharide, arachidonic acid (10^{-6}–10^{-4} M) dose-dependently reduced inducible nitric oxide synthase and cyclooxygenase-2 activity as well as reducing the expression of both enzyme proteins. Indomethacin (10^{-7}–10^{-4} M) suppressed prostaglandin E_2 release into the medium and reduced cyclooxygenase-2 activity irreversibly, as expected, but the amount of cyclooxygenase-2 protein was increased (because indomethacin removes the inhibitory effect of prostaglandins on cyclooxygenase-2 expression). However, the same doses of indomethacin also inhibited the release of nitric oxide into the medium. This was accompanied by a reduction in the amount of inducible nitric oxide synthase protein. The drug was not cytotoxic. Thus these results show that pharmacological treatments can have opposing actions on inducible nitric oxide synthase and cyclooxygenase-2 induction, with indomethacin showing an additional unexpected action in macrophages which may diminish their cytotoxic potential.

Keywords: Nitric oxide synthase, inducible (NO); Cyclooxygenase-2; Western blot; Lipopolysaccharide; cAMP; Indomethacin; Cytotoxicity

1. Introduction

Activation of macrophages by bacterial endotoxin (lipopolysaccharide) leads to the generation of greatly increased amounts of nitric oxide (NO) and eicosanoids (Stuehr and Marletta, 1987; Masferrer et al., 1990; Salvemini et al., 1993; Akarasereenont et al., 1994). This occurs after a lag of several hours and is due to the expression of the inducible isoforms of inducible nitric oxide synthase and cyclooxygenase-2 by a process which may be prevented by glucocorticoids. We showed recently that pretreatment with prostaglandin E_2 or other drugs which elevate cyclic AMP levels also prevents the induction in the mouse macrophage cell line J774 of both inducible nitric oxide synthase and cyclooxygenase-2 by lipopolysaccharide (Pang and Hoult, 1996). Arachidonic acid also reduced lipopolysaccharide-induced NO and prostaglandin E_2 generation and the amounts of inducible nitric oxide synthase and cyclooxygenase-2 protein, consistent with a role for endogenously formed eicosanoids in this process. We therefore expected indomethacin to be capable of blocking the down-regulation of these two enzymes by preventing the conversion of arachidonic acid to prostaglandins. However, we show here that indomethacin at non-toxic doses partially prevents the induction by endotoxin of inducible nitric oxide synthase in J774 cells, whereas cyclooxygenase-2 expression is enhanced.

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2. Materials and methods

2.1. Cell culture; measurement of nitrite, prostaglandin E_2 released into the medium and assessment of cyclooxygenase activity

J774.2 cells from ECACC (Porton Down, UK) were cultured at 5×10^5 cells per well in 24-well plates in 1.0 ml Dulbecco's modified Eagle's medium containing 5% fetal calf serum, 100 IU/ml penicillin and 100 μg/ml streptomycin. Following a 2 h pretreatment with indomethacin and/or arachidonic acid, the cells were cultured at 37°C in 5% CO_2 for 16 h with 500 ng/ml *E. coli* lipopolysaccharide (serotype 026:B6, Sigma). The culture medium was removed for assay of NO generation (as nitrite), quantified by adding 100-μl aliquots of medium to 100 μl of Griess reagent (1% sulphanilamide, 0.1% naphthylethyldiamine in 5% phosphoric acid), and comparing the resulting OD_{550} values in a microplate reader to those obtained from a standard curve of sodium nitrite also prepared in medium.

These samples of culture medium were also retained for measurement of the amount of prostaglandin E_2 released into the medium. This was done by direct radioimmunoassay of 100 μl aliquots, added to 200 μl polyclonal rabbit anti-PGE_2 serum diluted 1:1000 and 100 μl [$^3H_8$]PGE_2 containing 10 nCi (17 pg) (Amersham, sp. act. 181 Ci/mmol) for 18 h at 4°C, with bound label separated after centrifugation from free using 200 μl dextran-coated charcoal.

Cyclooxygenase activity was measured functionally by washing the cells three times in phosphate-buffered saline and then adding 10^{-6} M arachidonic acid for a further 1 h incubation in culture medium. Culture materials were from Life Technologies, Gibco BRL (Paisley, UK) and chemicals were from Sigma-Aldrich (Poole, UK).

2.2. Cell viability assay

Cell viability was measured by adding 50 μl 5 mg/ml thiazolyl blue (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, or MTT) to cells in 24 well plates and incubating for 1 h at 37°C. After removing the medium, 500 μl dimethylsulphoxide was added to solubilize the blue coloured tetrazolium and the plates were shaken for 5 min. Aliquots of 200 μl were transferred to 96-well plates and the OD_{550} values were read in a microplate reader. Viability was set as 100% in control cells.

2.3. Western blotting

Expression of inducible nitric oxide synthase and cyclooxygenase-2 proteins was measured by Western blotting as described previously (Pang et al., 1996). The density of bands corresponding to the position of inducible nitric oxide synthase (130 kDa) and cyclooxygenase-2 (70 kDa) was measured using a densitometer scanner and MCID software (Imaging Research, Canada).

Table 1
Effect of indomethacin and arachidonic acid on nitric oxide synthase and cyclo-oxygenase activity in J774 cells stimulated with lipopolysaccharide

Treatment	Inducible nitric oxide synthase activity (nitrite in medium after 16 h, μM)	PGE_2 release (amount in medium after 16 h, ng/ml)	Cyclooxygenase-2 activity (PGE_2 generated from 10^{-6} M AA in 1 h, ng/ml)	Cell viability (MTT assay, cells alone set as 100%)
Cells alone	1.08 ± 0.14 (24)	0.92 ± 0.16 (17)	2.46 ± 0.22 (13)	100.00 ± 0.00 (4)
LPS 500 ng/ml	15.04 ± 2.57 [a] (28)	4.88 ± 1.90 [a] (25)	7.59 ± 0.44 [a] (13)	68.62 ± 0.96 [a] (4)
+ AA 10^{-6} M	14.09 ± 0.86 (9)	9.74 ± 1.80 (9)	6.62 ± 0.42 (9)	not tested
+ AA 10^{-5} M	12.62 ± 2.67 [b] (9)	17.00 ± 1.99 (9)	6.00 ± 0.15 [c] (9)	not tested
+ AA 10^{-4} M	8.14 ± 0.70 [d] (9)	35.56 ± 6.28 (9)	3.32 ± 0.33 [d] (9)	not tested
+ indomethacin 10^{-7} M	14.61 ± 2.15 (7)	1.04 ± 0.04 [d] (4)	5.65 ± 0.21 [c] (4)	72.58 ± 2.22 (4)
+ indomethacin 10^{-6} M	13.24 ± 0.81 [b] (13)	0.85 ± 0.10 [d] (21)	3.61 ± 0.15 [d] (12)	64.79 ± 0.75 (4)
+ indomethacin 10^{-5} M	11.36 ± 0.65 [c] (11)	0.45 ± 0.03 [d] (4)	3.85 ± 0.17 [d] (4)	61.69 ± 1.59 (4)
+ indomethacin 10^{-4} M	8.52 ± 0.55 [d] (7)	0.40 ± 0.01 [d] (4)	2.18 ± 0.12 [d] (4)	75.78 ± 1.72 (4)
+ AA 10^{-6} M/indo 10^{-6} M	8.24 ± 1.36 [c] (9)	1.10 ± 0.23 [d] (6)	3.67 ± 0.28 [d] (8)	not tested
+ AA 10^{-5} M/indo 10^{-6} M	7.75 ± 1.42 [d] (9)	0.96 ± 0.14 [d] (9)	3.48 ± 0.30 [d] (9)	not tested
+ AA 10^{-4} M/indo 10^{-6} M	3.63 ± 0.66 [d] (9)	3.53 ± 0.22 (6)	3.78 ± 0.38 [d] (9)	not tested

Abbreviations: AA = arachidonic acid, indo = indomethacin, MTT = thiazolyl blue, PGE_2 = prostaglandin E_2. Results show mean ± S.E.M. for (n) tests per treatment, performed in most cases on two or more separate batches of cells

[a] Statistically significant effect of lipopolysaccharide (LPS) treatment ($P < 0.001$).

[b] Statistically significant changes with respect to LPS-treated cells. $P < 0.05$. (Student's unpaired t-test).

[c] Same for $P < 0.01$.

[d] Same for $P < 0.001$.

3. Results

As expected, lipopolysaccharide caused a large increase in activity of nitric oxide synthase (release of nitrite) and cyclooxygenase (measured both as prostaglandin E_2 released into the medium and as conversion of arachidonic acid to prostaglandin E_2) (Table 1) and this was accompanied by the expression of inducible nitric oxide synthase and cyclooxygenase-2 protein in the J774 macrophages (Fig. 1, lane 2; Fig. 2, lane 2). Lipopolysaccharide also caused a small but significant loss of viability of the cells after 16 h culture, as determined by assay of mitochondrial oxidative function (Table 1).

Treatment of the J774 cells with arachidonic acid 2 h prior to lipopolysaccharide caused a dose-dependent release of large amounts of prostaglandin E_2 into the medium over the 16 h period of incubation (Table 1). However, this was accompanied by a significant decrease in NO generation and of cyclooxygenase activity (conversion of arachidonic acid to prostaglandin E_2, after washing the cells free of medium following the incubation). As well as this, there was a substantial reduction in the amounts of the inducible nitric oxide synthase and cyclooxygenase-2 proteins as demonstrated by Western blotting (Fig. 1A, lanes 5–7; and Fig. 2A, lane 4). These results may be explained in terms of the conversion of arachidonic acid to prostaglandins prior to and during the addition of lipopolysaccharide, which then act to down-regulate the expression of inducible nitric oxide synthase and cyclooxygenase-2 proteins normally induced by the endotoxin.

Addition of indomethacin (10^{-7}–10^{-4} M) 2 h prior to lipopolysaccharide caused dose-dependent abolition of prostaglandin E_2 release (to a level below that of unstimulated cells) (Table 1). Indomethacin also completely suppressed cyclooxygenase enzyme activity (despite washing the cells three times before adding arachidonic acid), showing that its effect is irreversible (Quellet and Percival, 1995). However, indomethacin also reduced the amount of NO released into the medium (up to 46.7% at 10^{-4} M, Table 1, $P < 0.001$) and this was accompanied by a small but significant reduction in the amount of inducible nitric oxide synthase protein (blots shown in Fig. 1A, lane 3, and Fig. 2, lane 3; quantitative analysis in Fig. 2B, $P < 0.05$). In contrast, indomethacin did not reduce the level of

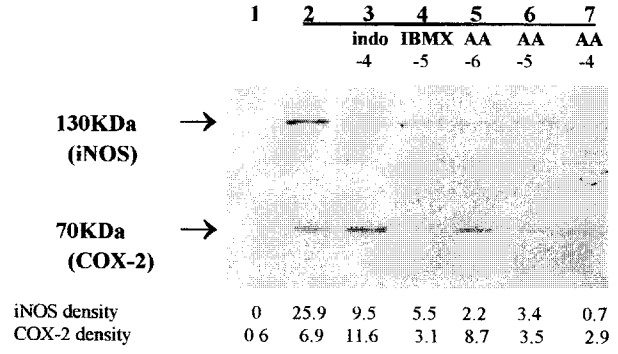

Fig. 1. Induction and repression of inducible nitric oxide synthase and cyclooxygenase-2 in J774 cells; effect of pretreatment with arachidonic acid. Lanes 1 and 2 show control untreated cells and those treated for 16 h with 500 ng/ml lipolysaccharide (LPS). All other lanes show cells treated with 500 ng/ml LPS together with 2 h pretreatments as shown at the top of each lane. Lane 3, pretreated with indomethacin at 10^{-4} M; lane 4, pretreated with isobutylmethylxanthine at 10^{-5} M; lanes 5–7, pretreated with arachidonic acid at 10^{-6} M, 10^{-5} M and 10^{-4} M, respectively. The positions and molecular masses of inducible nitric oxide synthase and cyclooxygenase-2 were validated by reference to molecular mass markers (Rainbow™, Amersham).

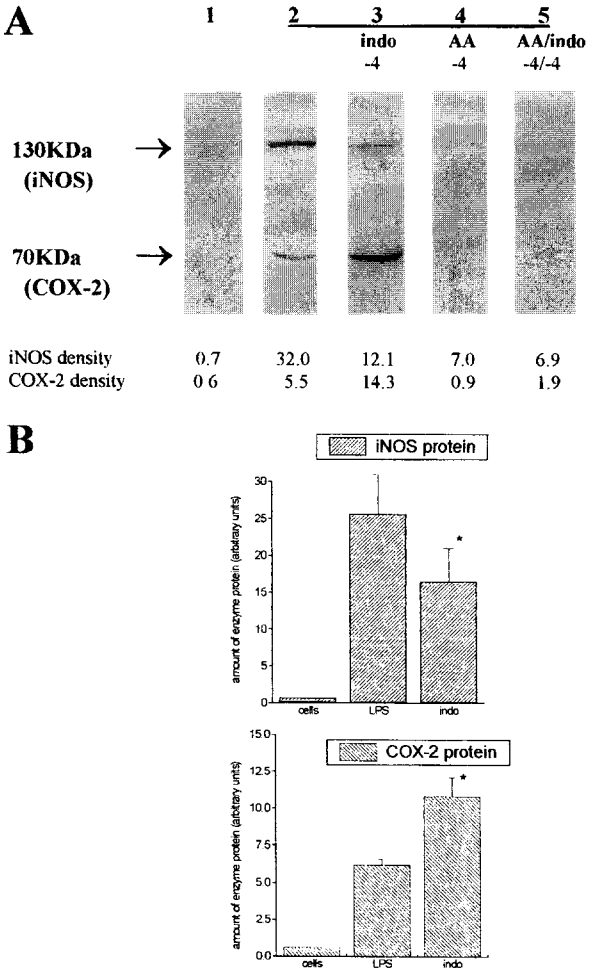

Fig. 2. (A) Effect of indomethacin on the induction by LPS of inducible nitric oxide synthase and cyclooxygenase-2 in J774 cells. Lane 1, control cells cultured for 16 h; lane 2, 500 ng/ml LPS for 16 h; lane 3, 10^{-4} M indomethacin added 2 h before 500 ng/ml LPS; lane 4, 10^{-4} M arachidonic acid added 2 h before 500 ng/ml LPS; lane 5, 10^{-4} M arachidonic acid and 10^{-4} M indomethacin added 2 h before 500 ng/ml LPS. (B) Densitometric quantitation of the effect of indomethacin pretreatment on the amounts of inducible nitric oxide synthase and cyclooxygenase-2 proteins. Values show mean ± S.E.M. from 5 independent experiments. * Indicates statistically significant effect of indomethacin, $P < 0.05$.

cyclooxygenase-2 protein. This was significantly increased (Fig. 1, lane 3, and Fig. 2, lane 3; $P < 0.05$). This latter effect of indomethacin to enhance cyclooxygenase-2 expression after lipopolysaccharide treatment is consistent with the removal of the inhibitory effect of prostaglandins, as mentioned above.

Given these results, it would therefore be expected that the combined addition of indomethacin with arachidonic acid 2 h prior to addition of lipopolysaccharide might prevent the down-regulation of the inducible nitric oxide synthase and cyclooxygenase-2 activities caused by the arachidonic acid. A concentration of 10^{-6} M indomethacin was sufficient to totally suppress the direct conversion of 10^{-6}–10^{-4} M arachidonic acid to prostaglandin E_2 (Table 1). It also partially prevented the down-regulation of cyclooxygenase-2 protein by the arachidonic acid as revealed by Western blotting (Fig. 2A, lane 5), although functionally this was masked by the irreversible inhibition of the enzyme (Table 1).

However, the combined treatment of indomethacin plus arachidonic acid did not reverse the inhibitory effects of arachidonic acid upon expression of inducible nitric oxide synthase. Instead, release of NO was inhibited to a greater extent (Table 1) and expression of inducible nitric oxide synthase protein remained attenuated (Fig. 2, lane 5).

Taken together, these data show that indomethacin exerts an inhibitory effect on the expression of inducible nitric oxide synthase induced by lipopolysaccharide and that this overrides any release from suppression that removal of endogenous eicosanoids might be expected to allow. That these effects of indomethacin are not due to any cytotoxic action is clearly shown from data in Table 1: although as mentioned lipopolysaccharide itself exerts a weak cytotoxic action (perhaps due to endogenous NO generation), the combination of lipopolysaccharide plus the various concentrations of indomethacin is not different. Moreover, cell viability remains 100% if the same range of doses of indomethacin is incubated for 16 h with J774 cells alone in the absence of lipopolysaccharide (data not shown).

4. Discussion

These results provide further evidence about the feedback negative modulatory role of prostaglandins on the induced nitric oxide synthase and cyclooxygenase enzymes in J774 mouse macrophages.

Previously, we found that high concentrations of prostaglandin E_2 or arachidonic acid added prior to lipopolysaccharide dose-dependently prevented the expression of inducible nitric oxide synthase and cyclooxygenase-2 proteins (Pang and Hoult, 1996) and that this effect could be produced also by 3 agents which enhance intracellular cyclic AMP activity (forskolin, isobutylmethylxanthine and 8-bromo-cyclic AMP). Although the ability of prostaglandin E_2 and cyclic AMP stimulants to negatively modulate inducible nitric oxide synthase induction in various cell types has been described before (Marotta et al., 1992; Bulut et al., 1993; Raddassi et al., 1993; Tetsuka et al., 1994; Amin et al., 1995), this had not been documented for cyclooxygenase-2. It therefore seems as if the two very different enzyme pathways share some common control features.

However, the present results make clear that pharmacological separation between the induction of inducible nitric oxide synthase and cyclooxygenase-2 can be achieved. Indomethacin enhanced the expression of cyclooxygenase-2 in lipopolysaccharide-treated cells, whereas inducible nitric oxide synthase expression and activity was reduced. The increased expression of cyclooxygenase-2 protein can be accounted for as a result of the release from suppression due to the absence of endogenously formed prostaglandins. Moreover, in human airway smooth muscle cells (which express cyclooxygenase-2 but not inducible nitric oxide synthase in response to cytokines), pretreatment with indomethacin completely inhibited prostaglandin E_2 release and cyclooxygenase activity but further enhanced cyclooxygenase-2 induction in response to interleukin-1β (Pang and Knox, unpublished experiments). Taken together, these findings all point to the conclusion that prostaglandins can negatively modulate the expression of the cyclooxygenase-2 enzyme, thereby mitigating against prostaglandin overproduction. There is an interesting parallel with the L-arginine:NO system, in that nitric oxide appears to inhibit the transcription of the inducible nitric oxide synthase gene in macrophages (Weisz et al., 1996) and NO shows direct feedback inhibition of the nitric oxide synthase enzyme in J774 cells (Assreuy et al., 1993).

That indomethacin partially prevented inducible nitric oxide synthase expression was surprising, but there is literature precedent that aspirin-like drugs may affect the expression of inducible nitric oxide synthase. For example, in a rat alveolar macrophage cell line stimulated with lipopolysaccharide and interferon-γ, it was found that pretreatment with 25–1000 μM indomethacin reduced NO release and cellular nitric oxide synthase enzyme activity as well as blunting the expression of inducible nitric oxide synthase mRNA (Aeberhard et al., 1995). It thus seems as though indomethacin is capable of interfering with the functional expression of the inducible nitric oxide synthase gene, although this is not a non-specific action as we find that this drug does not similarly affect cyclooxygenase-2 expression and it is not cytotoxic. The effect is not related to cyclooxygenase inhibition, as it is weaker and occurs at high doses, above those needed to suppress endogenous or arachidonic acid-derived prostaglandin E_2 synthesis (Table 1).

We also have preliminary evidence that piroxicam (a structurally dissimilar non-steroidal anti-inflammatory drug and cyclooxygenase-1/cyclooxygenase-2 inhibitor) also reduces NO generation (to 12.9 ± 0.4 μM at 10^{-6} M and

10.4 ± 0.6 μM at 10^{-5} M), but we have not tested its effect on inducible nitric oxide synthase expression in this system. However, in contrast, Amin et al. (1995) found that although high concentrations of aspirin and sodium salicylate reduced inducible nitric oxide synthase protein and mRNA expression in lipopolysaccharide-stimulated RAW264.7 mouse macrophages, this property was not shared by indomethacin. Thus the ability of indomethacin to interfere with the L-arginine:NO system in macrophages appears to vary with the precise experimental conditions. However, this additional action of indomethacin (albeit at doses larger than required to inhibit cyclo-oxygenase activity) may serve to diminish the cytotoxic potential of macrophages.

In summary, prostaglandins generated endogenously from arachidonic acid or added exogenously may under certain circumstances act as negative feedback modulators for the induction of inducible nitric oxide synthase and cyclooxygenase-2, key enzymes for two pathophysiologically important bioregulator pathways. This implies that the induction of inducible nitric oxide synthase and cyclo-oxygenase-2 shares common regulatory features. However, pharmacological discrimination appears to be possible in view of the opposing effects of indomethacin, as shown here. It remains to be seen whether indomethacin has a specific effect on the regulatory elements which control transcription of the inducible nitric oxide synthase gene.

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