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Pyrimidinoceptor potentiation of macrophage PGE_2 release involved in the induction of nitric oxide synthase

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1 We have previously demonstrated that Ca^{2+}/calmodulin-dependent protein kinase (CaMK) mediates pyrimidinoceptor potentiation of LPS-elicited inducible nitric oxide synthase (iNOS) induction in murine J774 macrophages. In the present paper, we have explored the role of cyclo-oxygenase (COX)-dependent prostaglandin E_2 (PGE_2) formation in this event.

2 In J774 macrophages predominantly expressing $P2Y_6$ receptors, the simultaneous addition of UTP and lipopolysaccharide (LPS) resulted in potentiated increase in PGE_2 release.

3 UTP-induced increased PGE_2 release was demonstrated by a concomitant increase in COX-2 protein expression, and was decreased by inhibitors specific for phosphatidylinositide-phospholipase C (PI-PLC), CaMK, protein kinase C (PKC), nuclear factor-kappa B (NF-κB) or COX-2.

4 NS-398 (a selective COX-2 inhibitor) reduced LPS plus UTP-elicited iNOS induction and nitrite accumulation, supporting for the positive regulation of iNOS gene expression by endogenous PGE_2.

5 Moreover, the cyclic AMP/PKA-dependent up-regulation of iNOS expression mediated by PGE_2 was drawn from the inhibitory effects of 2′,5′-dideoxyadenosine, KT5720 and H-89. Exogenous PGE_2 induced NF-κB activation and potentiated nitrite accumulation in response to LPS.

6 In addition to COX-2 induction, arachidonic acid (AA) release and steady-state mRNA levels of type V secretory phospholipase A_2 ($sPLA_2$) and Ca^{2+}-independent PLA_2 ($iPLA_2$) were also increased in the presence of LPS and UTP; the LPS-induced increase in $iPLA_2$ activity was also potentiated by UTP.

7 Taken together, we conclude that UTP-mediated COX-2 and $iPLA_2$ potentiation and PGE_2 formation contribute to the iNOS induction, and that CaMK activation is the primary step in the UTP enhancement of COX-2 induction.

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Keywords: Pyrimidinoceptor; CaMK; NF-κB; iNOS; COX-2; PGE_2; PLA_2; macrophages

Abbreviations: AA, arachidonic acid; AP-1, activator protein-1; CaMK, Ca^{2+}/calmodulin-dependent protein kinase; COX, cyclo-oxygenase; CREB, cyclicAMP-responsive element binding protein; DMEM, Dulbecco's modified Eagle's medium; EMSA, electrophoretic mobility shift assay; iNOS, inducible nitric oxide synthase; IP, inositol phosphate; $iPLA_2$; Ca^{2+}-independent phospholipase A_2; LPS, lipopolysaccharide; α,β-MeATP, α,β-methylene-ATP; 2MeSATP, 2-methylthio-ATP; NF-IL-6, nuclear factor for interleukin-6; NF-κB, nuclear factor-kappa B; PC, phosphatidylcholine; PDTC, pyrrolidine dithiocarbamate; PG, prostaglandin; PI, phosphatidylinositide; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; RT–PCR, reverse transcription-polymerase chain reaction; $sPLA_2$, secretory PLA_2; TNF-α, tumour necrosis factor α

Introduction

Nitric oxide (NO), a bio-active free radical, is involved in various physiological and pathological processes in many organ systems (Nathan, 1992). Recent studies have shown that the microbicidal and tumoricidal activity of activated macrophages is associated with the production of NO (Moncada *et al*., 1991; Nathan, 1992), which is formed from the reaction catalyzed by inducible NO synthase (iNOS) using L-arginine as substrate (Moncada *et al*., 1991). It has become increasingly evident that the release of arachidonic acid (AA) and the production of eicosanoids are early events in macrophage activation by inflammatory stimuli. Cyclo-oxygenase (COX) is the rate-limiting enzyme in the biosynthesis of eicosanoids. In addition to the well-characterized constitutive form, COX-1, an inducible form, COX-2, which is rapidly induced in inflammatory states, may provide the prostanoids involved in immune and/or inflammatory responses.

Several studies have demonstrated that many effectors of NO production lead to the simultaneous release of eicosanoids. For example, iNOS and COX-2 may be co-induced in macrophages by LPS (Salvemini *et al*., 1993). The existence of a cross-interaction between the physiological responses mediated by the NOS and COX systems has also been noted. In terms of the effect of COX-related metabolites on NO production, several studies have proposed a modulatory role of prostaglandin E_2 (PGE_2) on iNOS induction and enzyme activity. Cell type- and stimulus-dependent stimulation or inhibition of iNOS-dependent NO generation by COX-dependent mediators have both been observed. For example, PG activation of iNOS induction has been seen in peritoneal macrophages (Habib *et al*., 1994), J774 macrophages (Iwabuchi *et al*., 1997; Lin *et al*., 1999) and vascular smooth muscle cells (Koide *et al*., 1993), while PG inhibition of iNOS has been seen in mesangial cells (Tetsuka *et al*., 1994), peritoneal macrophages (Raddassi *et al*., 1993) and J774 macrophages (Marotta *et al*., 1992; Raddassi *et al*., 1993). These paradoxical results, sometimes seen in the same cell type, suggest that the complex and multiple signalling mechanisms are involved in iNOS regulation and as targets for PGE_2 action. The actual mechanisms responsible for these paradoxical results are currently unclear, but one possible explanation might be a biphasic effect of PGE_2, as lower

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concentrations of PGE_2 (0.1–20 nM) enhance, and higher concentrations (10–100 μM) reduce, NO production in J774 macrophages (Marotta *et al*., 1992; Iwabuchi *et al*., 1997; Pang & Hoult, 1997).

Nucleotides are released from activated platelets, mechanically stimulated endothelial cells, neurons, astrocytoma cells and injured cells at inflammatory sites (Boarder & Hourani, 1998). Several recent studies have provided evidence supporting the regulatory role of nucleotides on the immune systems (Tonetti *et al*., 1994; 1995). It is known that all the diverse functions regulated by nucleotides are mediated by P2 purinoceptors, which consist of a family of ion channel P2X receptors and a family of G protein-coupled P2Y receptors (Boarder & Hourani, 1998). Thus far, five mammalian P2Y receptor subtypes have been identified (Boarder & Hourani, 1998). $P2Y_1$ receptors respond selectively to ADP, while ATP may act as an antagonist. ATP and UTP are equally effective agonists at $P2Y_2$ receptors. $P2Y_4$ and $P2Y_6$ are two pyrimidine receptors and selective for UTP and UDP. $P2Y_{11}$ receptors has been recently described as a functional receptor that is selective for ATP. In the murine macrophage cell lines J774 and RAW 264.7, we have demonstrated the presence of a G protein-coupled P2Y pyrimidine receptor responsible for the dramatic effects of UTP on these cells. Activation of UTP-specific macrophage pyrimidine receptors results in the increased phosphoinositide (PI) turnover, activation of protein kinase C (PKC), an increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), the release of AA and leukotrienes, and intracellular acidification (Lin & Lee, 1996; Lin, 1997; Lin & Chen, 1997; Lin *et al*., 1998). In addition, we have recently shown that UTP is the most effective nucleotide in potentiating the effect of LPS on iNOS induction and NO production in mouse J774 macrophages. This potentiation requires a Ca^{2+}/calmodulin-dependent protein kinase (CaMK)-dependent pathway linked to the activation of transcription factor NF-κB (Chen *et al*., 1998). These observations led us to investigate whether UTP and other nucleotide analogues might play a role in the modulation of COX-2 induction and PGE_2 biosynthesis and, if this is the case, to study the underlying mechanism, the contribution of PLA_2 activity and the involvement of released PGE_2 in NO synthesis.

Methods

Cell culture

The mouse J774 macrophage cell line was obtained from American Type Culture Collection and grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal bovine serum and antibiotics (100 U ml^{-1} of penicillin and 100 μg ml^{-1} of streptomycin). Cells cultured at 37°C in a water-saturated atmosphere of 95% air and 5% CO_2 were seeded into 24-well plates for the nitrite and PGE_2 assay, into 10 cm dishes for immunoblots, or into 60 mm dishes for NF-κB activation and RT–PCR analysis.

Nitrite and PGE_2 measurement

For iNOS and COX-2 induction, fresh culture medium containing LPS and/or UTP at the concentrations indicated, was added to cells, then the nitrite and PGE_2 released into the medium measured 24 h later. Nitrite, an indicator of NO synthesis, was measured by adding 100 μl of Griess reagent (1% sulphanilamide and 0.1% naphthylethylenediamide in 5% phosphoric acid) to 100 μl samples of culture medium, then the optical density at 550 nm (OD_{550}) was measured using a microplate reader and the nitrite concentration calculated by comparison with the OD_{550} produced using standard solutions of sodium nitrite in culture medium. PGE_2 was measured using ELISA kit from Cayman, following the manufacturer's instruction.

Western blot analysis

To quantify iNOS, COX-2 and cytosolic phospholipase A_2 ($cPLA_2$) protein expression, following 24 h incubation in the presence of various agents, the cells were washed twice in ice-cold PBS, then solubilized in buffer containing (mM): Tris-HCl 20, EGTA 0.5, EDTA 2, DTT 2, PMSF 0.5 and 10 μg ml^{-1} of leupeptin (pH 7.5). Samples containing equal amounts of protein (100 μg) were subjected to SDS–PAGE on 7.5% (iNOS and $cPLA_2$) or 9% (COX-2) polyacrylamide gels, then transferred onto a nitrocellulose membrane, which was then incubated in Tris-buffered saline with 0.1% Tween-20 (TBST) containing 5% milk, and the iNOS, COX-2 and $cPLA_2$ bands visualized by immunoblotting with specific antibodies. Immunoreactivity was detected by enhanced chemiluminescence, following the manufacturer's instructions. Densitometrical analyses were performed on a Molecular Dynamics densitometer.

Preparation of nuclear extracts and electrophoretic mobility shift assay (EMSA)

Nuclear extracts from PGE_2-stimulated or non-stimulated cells were prepared as described previously (Chen *et al*., 1998). To detect NF-κB activation using the EMSA method, reaction mixtures (15 μl) contained 0.25 μg of poly (dI-dC) (Amersham Pharmacia Biotech), 20,000 d.p.m. of ^{32}P-labelled DNA probe, 10 mM Tris pH 7.5, 1 mM EDTA, 4% Ficoll, 1 mM DTT and 75 mM KCl; the binding reaction was started by addition of cell extracts and continued for 30 min. Samples were analysed on native 5% polyacrylamide gels.

^{3}H-AA release

AA release was measured as described previously (Lin & Chen, 1998). Briefly, cells were prelabelled with 0.3 μCi ml^{-1} of ^{3}H-AA in DMEM, then treated for 24 h at 37°C with 1 μg ml^{-1} of LPS, either alone or in combination with 100 μM UTP. The cells were washed three times with serum-free DMEM, then incubated in medium containing 0.5% fatty acid-free bovine serum albumin. After a 2 h incubation, the medium was removed and centrifuged at $250 \times g$ for 5 min to remove floating cells, then the radioactivity in the supernatant was measured.

RNA extraction and RT–PCR analysis of $sPLA_2$ and $iPLA_2$ mRNA

To amplify mouse type IIA, V secretory PLA_2 ($sPLA_2$) and Ca^{2+}-independent PLA_2 ($iPLA_2$) mRNA, the specific primers were synthesized. The type IIA $sPLA_2$ primers used were 5′-ATG AAG GTC CTC CTC CTG CTA G-3′ and 5′-TCA GCA TTT GGG CTT CTT CC-3′; the type V $sPLA_2$ primers were 5′-CAG GGG GCT TGC TAG AAC TCA A-3′ and 5′-AAG AGG GTT GTA AGT CCA GAG G-3′; and the $iPLA_2$ primers were 5′-AAC GTT AAC CTC AGG CCT CC-3′ and 5′-GAG AGT TTC TTC ACC TTG GTT-3′. β-actin mRNA levels were used as an internal control. The β-actin primers used were: sense (613–632), 5′-GAC TAC CTC ATG AAG

ATC CT-3′ and antisense (1101–1122), 5′-CCA CAT CTG CTG GAA GGT GG-3′. Confluent cells, grown in 100 mm Petri dishes, were treated with LPS ± UTP for different periods, then harvested. The total RNA was purified using RNAzol reagent, and RT–PCR carried out using a RNA PCR kit (Gibco), according to the manufacturer's instructions, using 10 μg of total RNA as a template. Equal amounts (1 μg cDNA) of each RT product were PCR-amplified using Taq polymerase in 30 cycles consisting of 1 min at 95°C, 1 min at 55°C and 2 min at 72°C. The amplified cDNA was then run on 1.5% (for IIA, V $sPLA_2$ and β-actin) or 2% (for $iPLA_2$) agarose gels and visualized by ethidium bromide. Relative changes in PCR products were normalized using the β-actin signal.

RT–PCR analysis of P2Y receptor subtypes

Total RNA was prepared as mentioned above and the P2Y primers used were: $P2Y_1$ receptor sense (661–680), 5′-ACG ACT GTG GCC ATG TTC TG-3′ and antisense (1051–1070), 5′-ATT TCT TCA CTC TTG GAT TG-3′; $P2Y_2$ receptor sense (31–50), 5′-ACC ATC AAT GGC ACC TGG GA-3′ and antisense (374–393), 5′-CCG GTG CAC GCT GAT GCA GG-3′; $P2Y_4$ receptor sense, 5′-CAC CGA TAC CTG GGT ATC TG-3′ and antisense, 5′-CAG ACA GCA AAG ACA GTC AG-3′, $P2Y_6$ receptor sense (315–334), 5′-GCT TCC TCT TCT ATG CCA AC-3′ and antisense (779–798), 5′-GTA GGC TGT CTT GGT GAT GT-3′; $P2Y_{11}$ receptor sense (94–113), 5′-CTG GTG GTT GAG TTC CTG GT-3′ and antisense (308–327), 5′-GTT GCA GGT GAA GAG GAA GC-3′. Equal amounts of each RT product were PCR-amplified with Taq polymerase in 30 cycles consisting of 40 s at 95°C, 40 s at 48°C (for $P2Y_1$ receptor), 54°C (for $P2Y_2$ receptor), 55°C (for $P2Y_4$ and $P2Y_6$ receptors) or 57°C (for $P2Y_{11}$ receptor) and 2 min at 72°C. The amplified cDNA was run on 1% agarose gels and visualized by ethidium bromide.

Assessment of PLA_2 activity

J774 cells in 1 ml of culture medium were seeded into 35 mm culture dishes and left until confluent, then the medium was replaced with 1 ml of fresh medium containing 1 μg ml^{-1} LPS either alone or in combination with 100 μM UTP for 24 h at 37°C. After 24 h of treatment, the supernatants were collected, and the cells incubated for a further 15 min at 37°C with 1 ml of culture medium containing 1 M NaCl, which allowed cell surface-associated $sPLA_2$ to be recovered in the medium without significant cell death, as assessed by the MTT test (data not shown). The remaining cells were harvested using a cell scraper, suspended in 500 μl of cell lysis buffer consisting of 10 mM HEPES, pH 7.5, 1 mM EDTA, 0.1 mM DTT, 0.34 M sucrose, and 1 μg ml^{-1} of leupeptin, and lysed by sonication (2×5 s); the resulting homogenate was centrifuged at $10{,}000 \times g$ for 20 min at 4°C, and the supernatant collected.

$sPLA_2$ activity in the final 1 ml culture supernatant or in the 1 M NaCl extract was assayed by measuring the amount of free linoleic acid released from 5 μM 1-palmitonyl-2-[^{14}C]linoleoyl-sn-glycero-3-phosphoethanolamine (56.0 mCi mmol^{-1}). The radioactive phospholipid was dried under nitrogen and sonicated in vesicle buffer consisting of 50 mM HEPES, pH 7.5 and 1 mM EGTA. Each reaction mixture consisted of an aliquot of the test sample, 100 mM Tris-HCl, pH 7.5, 4 mM $CaCl_2$, and 5 μM substrate and incubation was performed at 37°C for 30 min (Murakami *et al*., 1998).

$iPLA_2$ activity was determined by adding 30 μl of homogenate supernatant to 170 μl of Ca^{2+}-free buffer (mM): Tris-HCl 50, pH 7.5, EDTA 5, substrate 5 μM and 1 mg ml^{-1} of fatty acid free BSA at 37°C for 30 min (Larsson *et al*., 1998). The [^{14}C] linoleic acid released was extracted and counted as described previously (Murakami *et al*., 1998).

$cPLA_2$ activity was determined by adding 30 μl of homogenate supernatant to 170 μl of Ca^{2+}-containing assay buffer (mM): glycine 80, pH 9.0, $CaCl_2$ 5, dithiothreitol 2, 1 mg ml^{-1} of fatty acid free BSA and 5 μM 1-stearoyl-2-[^{14}C] arachidonyl-sn-glycero-3-phosphatidylcholine (55.0 mCi mmol^{-1}) at 37°C for 30 min (Larsson *et al*., 1998). The [^{14}C]AA released was determined.

Chemicals

DMEM, foetal bovine serum, penicillin and streptomycin were obtained from Gibco BRL (Grand Island, NY, U.S.A.). [α-^{32}P]-ATP (3,000 Ci mmol^{-1}), horseradish peroxidase-coupled anti-mouse, anti-rabbit antibodies, the enhanced chemiluminescence detection agent, 1-palmitonyl-2-[^{14}C]linoleoyl-sn-glycero-3-phosphoethanolamine and 1-stearoyl-2-[^{14}C]arachidonyl-sn-glycero-3-phosphatidylcholine were purchased from Amersham Pharmacia Biotech. Ro 31-8220, KN-93, NS-398, Go 6976, H-89 and KT5720 were purchased from Calbiochem (La Jolla, CA, U.S.A.). Genistein, 2-methylthio-ATP (2MeSATP) and α,β-methylene-ATP (α,β-MeATP) were from RBI (Natick, MA, U.S.A.). U73122, D609 and 2′,5′-dideoxyadenosine were from Biomol Research Laboratories Inc. (Plymouth Meeting, PA, U.S.A.). Rabbit polyclonal anti-iNOS antibody was purchased from Transduction Laboratories (Lexington, KY, U.S.A.). Antibodies directed against COX-2 and $cPLA_2$ were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.) The PGE_2 ELISA kit was purchased from Cayman. RNAzol was obtained from Biotecx Laboratories Inc (Houston, TX, U.S.A.). [^{3}H]AA (100 Ci mmol^{-1}) was purchased from New England Nuclear (Boston, MA, U.S.A.). Oligonucleotides were synthesized on a PS 250 CRUACHEM DNA synthesizer, using the cyanoethyl phosphoramidate method, and purified by gel filtration. The sequence of the double-stranded oligonucleotide used to detect NF-κB DNA-binding activity was 5′-GAT-CAGTTGAGGGGACTTTCCCAGGC-3′ (the binding site is underlined). All materials for SDS–PAGE were obtained from Bio-Rad Laboratories (Hercules, CA, U.S.A.). All other chemicals were obtained from Sigma (St. Louis, MO, U.S.A.).

Statistical analysis

Values are expressed as the mean ± s.e.mean of at least three experiments. Analysis of variance (ANOVA) and/or Dunnets test were used to assess the statistical significance of the differences, a P value less than 0.05 being considered statistically significant.

Results

Expression of $P2Y_2$, $P2Y_6$ and $P2Y_{11}$ receptors in J774 macrophages

Upon using RT–PCR analysis to study the expression of five known P2Y receptor subtypes in J774 macrophages, we detected the predominant mRNA signal of $P2Y_6$ receptor and weak mRNA signals of $P2Y_2$ and $P2Y_{11}$ receptors (Figure 1). $P2Y_1$ and $P2Y_4$ receptors, which were expressed respectively in bovine pulmonary artery endothelial cells (CPAE) and rat aortic smooth muscle cells (SMC), were not detected in J774 macrophages (Figure 1).

Pyrimidinoceptor-mediated potentiation of PGE_2 release and COX-2 induction

Incubation of J774 macrophages for 24 h with LPS (0.01–1 μg ml^{-1}) resulted in a concentration-dependent increase in PGE_2 levels from the basal value of 300 ± 75 to $3{,}395\pm375$ pg ml^{-1} ($n=6$) in the presence of 1 μg ml^{-1} LPS (Figure 2a). 100 μM UTP alone caused an approximately 2 fold increase in PGE_2 production ($n=4$), but, when added together with LPS and left in the medium for 24 h, markedly potentiated the LPS-induced response to $10{,}118\pm710$ pg ml^{-1} ($n=5$) at 1 μg ml^{-1} LPS (Figure 2b). The potentiating effect of UTP was also concentration-dependent in the range of 3–300 μM with an increase of $213\pm18\%$ ($n=3$) being seen at 300 μM UTP in the presence of a fixed concentration of 1 μg ml^{-1} LPS (Figure 2b). To ensure that the increased PGE_2 production resulted from increased COX-2 expression, COX-2 levels were determined by Western blotting of 24 h drug-treated cells. As shown in Figure 3, the levels of COX-2 induced by 0.3 and 1 μg ml^{-1} LPS were increased, respectively, by 73 ± 2 and $96\pm7\%$ ($n=3$) in the presence of 100 μM UTP, while UTP alone was unable to elicit COX-2 protein expression.

Next, we determined whether the increase in the LPS-induced PGE_2 response seen in the presence of UTP was also mediated by other nucleotide analogues. As shown in Table 1, comparison of the potency and efficacy of various nucleotides showed that 100 μM ATP (a non-selective P2 agonist) increased LPS-induced PGE_2 production by $124\pm15\%$ ($n=3$), while 2MeSATP (a preferential $P2Y_1$ and $P2Y_{11}$ receptor agonist), α,β-MeATP (a selective P2X receptor agonist) and adenosine (a selective P1 receptor agonist), all at 100 μM, produced no significant potentiation.

Roles of protein kinases and NF-κB in PGE_2 production

To explore the underlying mechanism involved in UTP-induced PGE_2 potentiation, the roles of PKC, tyrosine kinase and NF-κB were investigated using pharmacological approaches. As shown in Figure 4a, the PKC inhibitors, Ro 31-8220 (Davis *et al*., 1992) and Go 6976 (Martiny-Baron *et al*., 1993), dramatically attenuated not only the LPS-induced PGE_2 response, but also the potentiation effect of UTP. Ro 31-8220 (1 μM) and Go 6976 (100 nM) inhibited the LPS-induced response by $80\pm9\%$ ($n=4$) and $77\pm9\%$ ($n=3$), respectively,

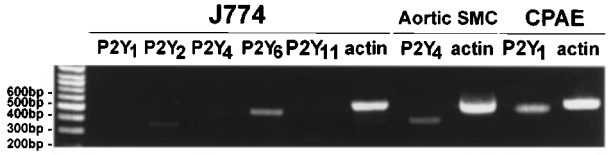

Figure 1 Analysis of P2Y receptor mRNA in J774 macrophages. Total RNA were prepared from J774 macrophages, bovine pulmonary artery endothelial cells (CPAE) and aortic smooth muscle cells (SMC), and then PCR analysis for five P2Y receptor subtypes and β-actin were performed as described in Methods.

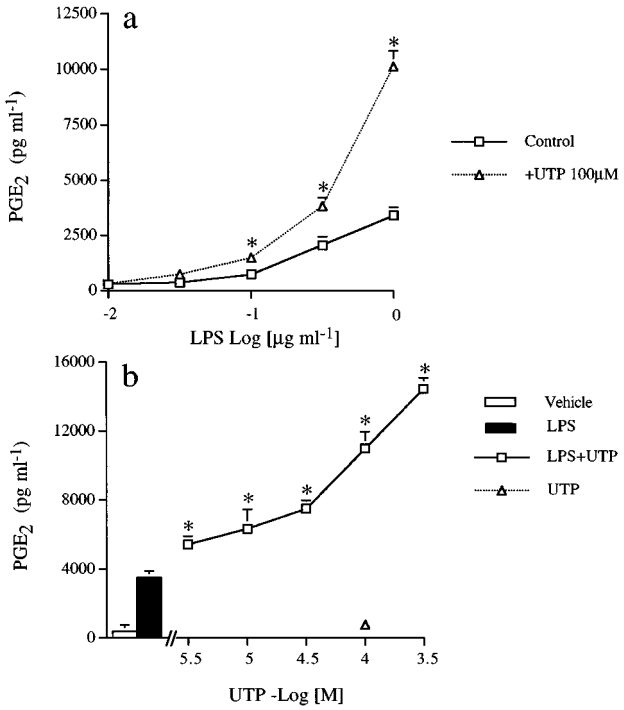

Figure 2 Concentration-dependent PGE_2 release by LPS- and LPS/UTP-stimulated J774 macrophages. (a) Cells were incubated for 24 h with different concentrations of LPS in the absence or presence of 100 μM UTP before assaying for PGE_2. (b) Cells were incubated for 24 h with vehicle, 100 μM UTP alone, or 1 μg ml^{-1} LPS in the absence or presence of different concentrations of UTP. The data represent the mean$\pm$s.e.mean of at least three experiments performed in duplicate. *$P<0.05$ compared with the control response to LPS.

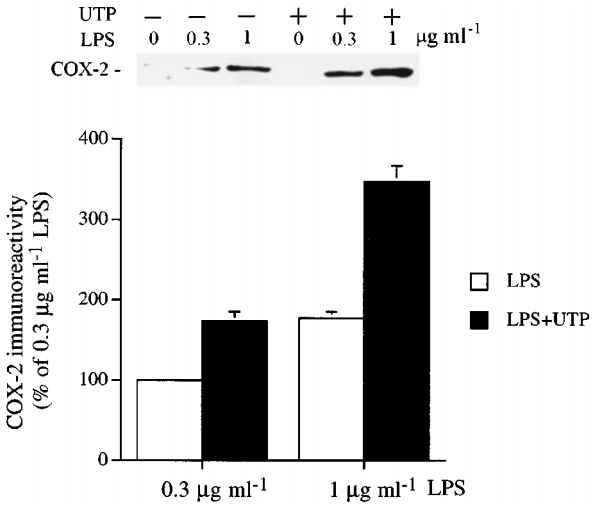

Figure 3 Effect of LPS and UTP on COX-2 expression. Cells were treated with vehicle or LPS (0.3–1 μg ml^{-1}) for 24 h, in the presence or absence of UTP (100 μM), then subjected to immunoblotting for COX-2 protein, as described in Methods. The typical traces are representative of three experiments. Band intensities were quantified using densitometric imaging software.

Table 1 Effects of nucleotide analogues and adenosine on the LPS-induced PGE_2 formation in J774 macrophages

Treatment	*PGE_2* (pg ml^{-1})
LPS alone	3065 ± 375
+ UTP (100 μM)	8885 ± 737
+ ATP (100 μM)	6885 ± 452
+ 2MeSATP (100 μM)	2850 ± 494
+ α,β-MeATP (100 μM)	3250 ± 378
+ Adenosine (100 μM)	2738 ± 250

The basal level of PGE_2 was 365 ± 50 pg ml^{-1}. Values are the mean$\pm$s.e.mean of 3–4 experiments.

and reduced the UTP-induced potentiation by $76 \pm 3\%$ ($n=4$) and $71 \pm 2\%$ ($n=3$), respectively. These results suggest that activated PKC is a crucial factor in the induction of PGE_2 production. Genistein (50 μM), a tyrosine kinase inhibitor, also completely abolished the PGE_2 response induced by LPS, either alone or in the presence of UTP (Figure 4a).

Since NF-κB activation is required for COX-2 induction (D'Acqisto *et al*., 1997) and UTP-induced NO potentiation (Chen *et al*., 1998), the involvement of this transcription factor in UTP enhancement of the LPS-induced PGE_2 increase was studied. As shown in Figure 4a, 50 μM pyrrolidine dithiocarbamate (PDTC), a NF-κB inhibitor, abolished PGE_2 formation induced by LPS, either alone or in combination with UTP.

Involvement of PI turnover and intracellular Ca^{2+} in the UTP-mediated response

To determine whether the potentiation effect of UTP on LPS-induced COX-2 expression depends on PI signalling cascades, the effect of the PI-PLC inhibitor, U73122, was tested. Figure 4b shows that 3 μM U73122 decreased UTP-induced PGE_2 potentiation without affecting the response to LPS alone; in contrast, 30 μM D609, a PC-PLC inhibitor, decreased the LPS-induced response by $67 \pm 16\%$ ($n=3$), but failed to affect the potentiation response of UTP, the increase being 222% and 260% of control in D609-treated and -untreated cells.

When cells were treated with 30 μM BAPTA/AM, an intracellular Ca^{2+} chelator, UTP potentiation of LPS-induced PGE_2 production was not seen (Figure 4b), suggesting that an increase in $[Ca^{2+}]_i$ was necessary for the UTP response. In this context, we recently showed that CaMK is involved in the UTP potentiation of LPS-induced NF-κB and AP-1 activation, and iNOS expression in J774 macrophages (Chen *et al*., 1998). Consistent with these findings, KN-93 (3–10 μM), a CaMK inhibitor, inhibited the potentiating effect of UTP on the LPS-induced increase in PGE_2 levels in a concentration-dependent manner (Figure 4b). These results suggest the involvement of the Ca^{2+}/CaMK pathway in pyrimidinoceptor-mediated potentiation of both iNOS and COX-2 expression in J774 macrophages.

Modulation of iNOS expression by endogenous and exogenous PGE_2

Synthesis of various gene products can be induced in macrophages; COX-2 is one such product, and the released PGE_2 could therefore be involved in the regulation of macrophage functions in an autocrine/paracrine fashion. In

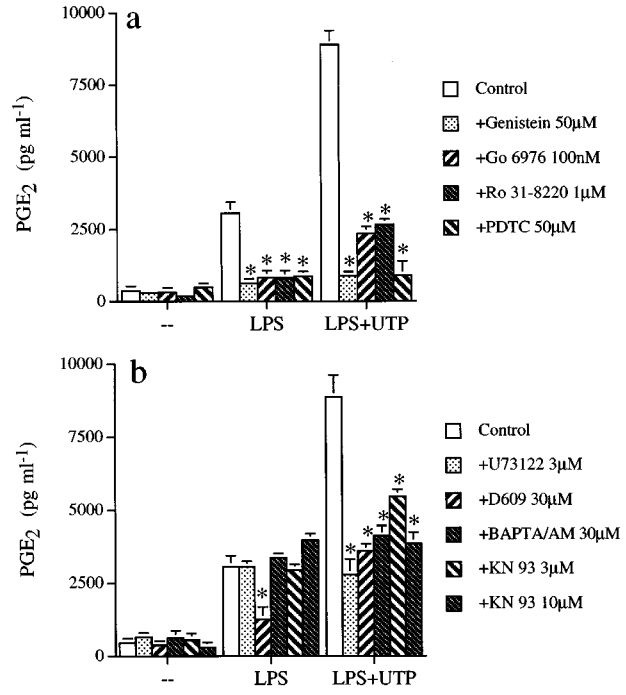

Figure 4 Effects of pharmacological inhibitors on LPS- and LPS/UTP-induced PGE_2 formation. Cells were incubated for 20 min with 50 μM genistein, 100 nM Go 6976, 1 μM Ro 31-8220, 50 μM PDTC, 3 μM U73122, 30 μM D609, 30 μM BAPTA/AM or 3 or 10 μM KN-93 before addition of LPS (1 μg ml^{-1}) with or without 100 μM UTP. After 24 h incubation, the amount of PGE_2 released into the medium was determined (a and b). The data represent the mean $\pm$ s.e.mean of 3–4 experiments performed in duplicate. $^*P<0.05$ compared with the control response (LPS $\pm$ UTP) without inhibitor treatment.

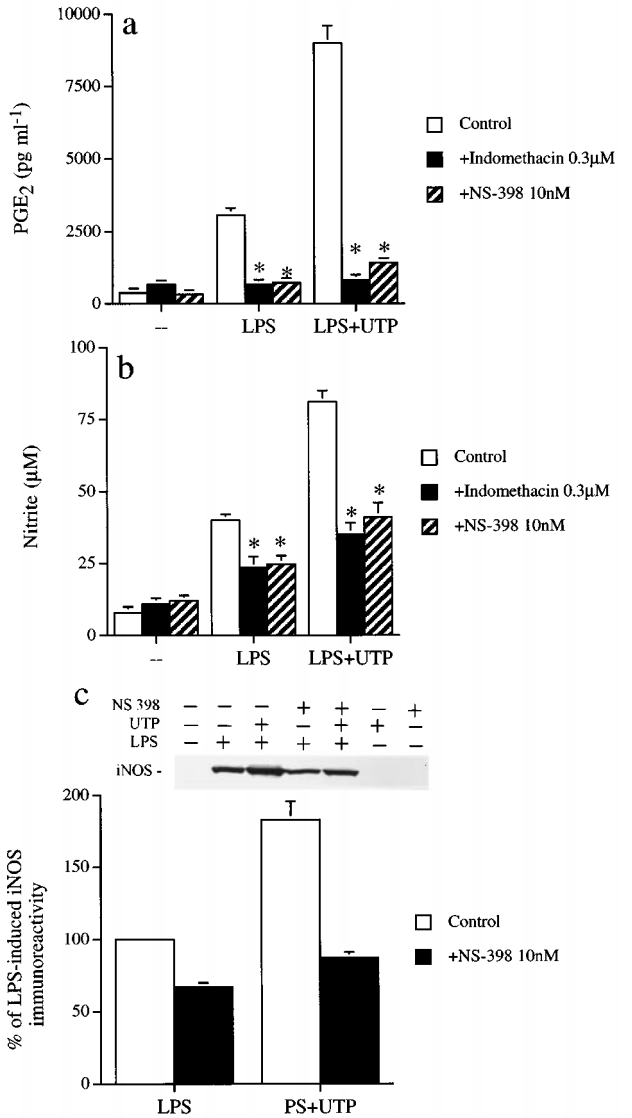

Figure 5 Effects of COX inhibitors on PGE_2, nitrite production and iNOS induction. J774 cells were preincubated for 20 min with indomethacin (0.3 μM) or NS-398 (10 nM), then incubated for 24 h in the presence of LPS (1 μg ml^{-1}) either alone or with UTP (100 μM) before assaying for PGE_2 (a) or nitrite (b) release or iNOS immunoreactivity (c). In (c), the typical iNOS immunoreactivity after overnight treatment with the various combinations of reagents is shown. $^*P<0.05$ compared to the control response (LPS $\pm$ UTP) without drug pretreatment. The data in (a) and (b) represent the mean $\pm$ s.e.mean of three experiments performed in duplicate and the data in (c) represent the average from three independent experiments.

murine J774 macrophages, we found that PGE_2 release induced by LPS ± UTP was dramatically inhibited by the non-selective COX inhibitor, indomethacin (0.3 μM), and the selective COX-2 inhibitor, NS-398 (10 nM) (Figure 5a). In parallel, LPS-induced nitrite accumulation within the same incubation period was 57% and 41% inhibited by the presence of these respective inhibitors, while the potentiation effect of UTP on nitrite accumulation was 68% and 76% inhibited by indomethacin and NS-398, respectively (Figure 5b). Consistently as shown in Figure 5c, addition of NS-398 led to a decrease in iNOS induction. In the presence of NS-398 (10 nM), LPS-induced iNOS protein expression was inhibited by 33 ± 3% ($n=3$), and the UTP potentiation effect decreased from 83 ± 13 to 30 ± 6% ($n=3$). These results indicate that endogenous PGE_2 displays not only as a positive regulator on NO formation but also as a mediator for UTP potentiation on NO production.

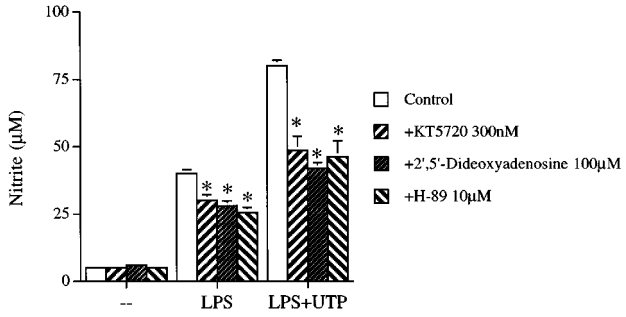

Figure 6 Effects of adenylyl cyclase and PKA inhibitors on UTP potentiation of NO formation. Cells were preincubated for 20 min with 2′,5′-dideoxyadenosine (100 μM), KT5720 (300 nM) or H-89 (10 μM), then incubated for 24 h in the presence of LPS (1 μg ml^{-1}) either alone or with UTP (100 μM) before assaying for nitrite release. *$P<0.05$ compared to the control response (LPS ± UTP) without drug pretreatment. The data represent the mean ± s.e.mean of four experiments performed in duplicate.

Next we explored whether the regulatory role of PGE_2 comes from cyclicAMP/protein kinase A (PKA) signalling. Treating cells with 100 μM 2′,5′-dideoxyadenosine (an adenylyl cyclase inhibitor), 300 nM KT5720 or 10 μM H-89 (two selective PKA inhibitors) reduced LPS-induced as well as UTP-potentiated NO formation (Figure 6). UTP-elicited NO potentiation was approximately 65 ± 6%, 54 ± 13% and 49 ± 15% ($n=4$) inhibited by the presence of 2′,5′-dideoxyadenosine, KT5720 and H-89, respectively.

To further confirm the positive regulatory role of endogenous PGE_2 on NO formation in J774 cells suggested from our present results, we directly added PGE_2 to the cells and found that exogenous PGE_2 (0.01 – 10 nM) potentiated LPS-induced nitrite accumulation in a concentration-dependent manner (Figure 7a), the increase at 10 nM being 78 ± 16% ($n=4$). Addition of PGE_2 in the absence of LPS did not trigger NO formation (data not shown). To assess the possible mechanism underlying this potentiating effect, we studied the effect of PGE_2 on NF-κB activation, a key and essential factor for iNOS gene transcription (Chen *et al*., 1998). EMSA of the nuclear proteins indicated that, over the same concentration range (0.1 – 10 nM) used to potentiate NO formation, PGE_2 resulted in the NF-κB activation after 30 min treatment (Figure 7b).

Effects of LPS and UTP on ^{3}H-AA release and PLA_2 expression

In addition to the COX-2 induction seen in parallel with the enhanced PGE_2 formation, we further determined whether the amount of AA, the precursor for PGE_2 biosynthesis, was altered by the presence of LPS plus UTP. As shown in Figure 8a, LPS treatment for 24 h resulted in a 52 ± 8% ($n=3$) increase in AA release, which was further increased to 92 ± 2% ($n=3$) by the co-addition of UTP (100 μM). UTP alone had no effect on endogenous PLA_2 activity at 24 h treatment. In the following study, we determined which PLA_2 isoform accounted for the increased AA release elicited by LPS and UTP.

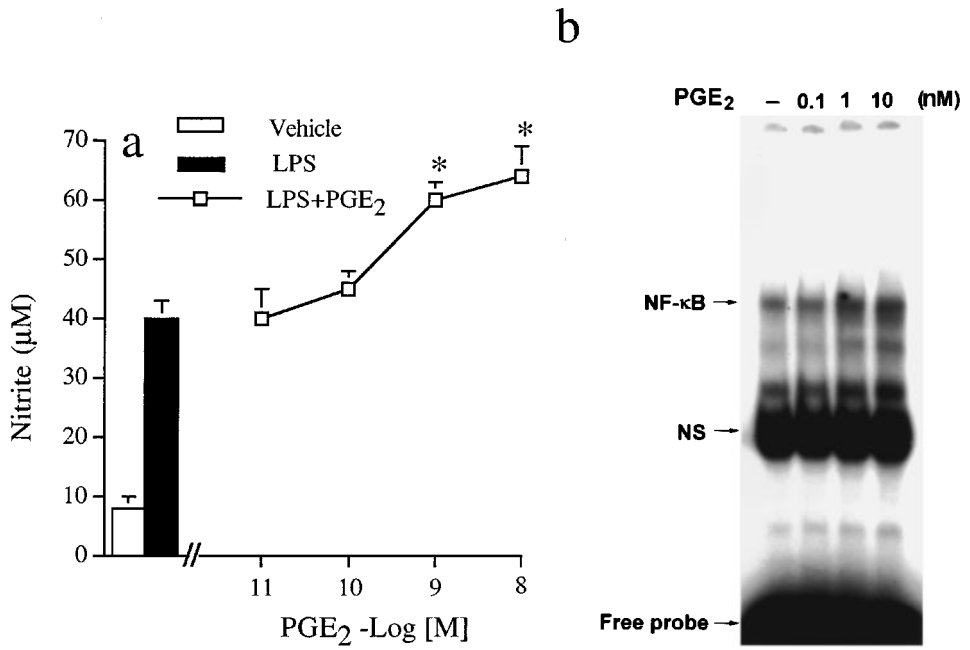

Figure 7 Concentration-dependent effect of PGE_2 on nitrite potentiation and NF-κB activation. (a) Cells were incubated for 24 h with vehicle or LPS (1 μg ml^{-1}) in the absence or presence of increasing concentrations of PGE_2 before assaying for nitrite. The data represent the mean ± s.e.mean of 3 – 4 experiments performed in duplicate. *$P<0.05$ compared with the control response to LPS. (b) Macrophages were incubated with PGE_2 (0.1 – 10 nM) for 30 min, then NF-κB activation was evaluated by EMSA as described in Methods. The results are representative of three different experiments.

Firstly, Western blot analysis using antibody specific for $cPLA_2$ revealed that protein levels of $cPLA_2$ were unaltered following LPS treatment, with or without UTP, for 24 h (Figure 8b). Secondly, using RT–PCR analysis, we measured basal levels and changes in mRNAs for type IIA and V $sPLA_2$, and $iPLA_2$ in J774 cells. In addition to $cPLA_2$, all these three PLA_2 isoforms have been demonstrated in macrophages (Ackermann *et al*., 1994; Naraba *et al*., 1998). As shown in Figure 8b, mRNA for type IIA, V, $sPLA_2$ and $iPLA_2$ were detected in control cells. Both type V $sPLA_2$ and $iPLA_2$ mRNA levels increased after 8 h treatment with LPS and this effect was potentiated by UTP co-addition. During 8 h treatment with LPS plus UTP, mRNA levels for type IIA $sPLA_2$ remained unchanged. Thirdly, using 1-palmitoyl-2-[^{14}C]linoleoyl-sn-glycero-3-phosphoethanolamine as a substrate, we directly measured the enzymatic activities of $sPLA_2$ and $iPLA_2$ in the medium, membrane and cytosol. As shown in Figure 9, we found that LPS treatment for 24 h resulted in a $79 \pm 17\%$ ($n=3$) increase in $iPLA_2$ activity, which was further increased to $173 \pm 36\%$ ($n=3$) in combination with 100 μM UTP (Figure 9c). However, neither secreted or membrane-solubilized $sPLA_2$ was affected by LPS alone or in combination with UTP (Figure 9a,b). On the other hand, using 1-stearoyl-2-[^{14}C]arachidonyl-sn-glycero-3-phosphatidylcholine as the $cPLA_2$ substrate, we did not detect any significant change by the treatment with LPS and/or UTP (Figure 9d). Taken together, these results demonstrate that $iPLA_2$ is the major enzyme responsible for the enhanced AA release seen with LPS and UTP.

Discussion

The present study shows that UTP can potentiate LPS-induced COX-2 protein expression, thereby enhancing PGE_2 release. The fact that PGE_2 release was inhibited by NS-398 (a COX-2 inhibitor) indicates that PGE_2 was *de novo* biosynthesized *via* COX-2 induction. In addition, UTP alone could not elicit COX-2 induction, but required the presence of LPS, suggesting that the signalling cascade triggered by UTP displays a regulatory role on COX-2 protein expression, while itself is not sufficient for this effect.

In terms of the underlying signalling mechanism responsible for UTP effect, our data also provide strong evidence for a requirement for pyrimidinoceptor-mediated PI turnover,

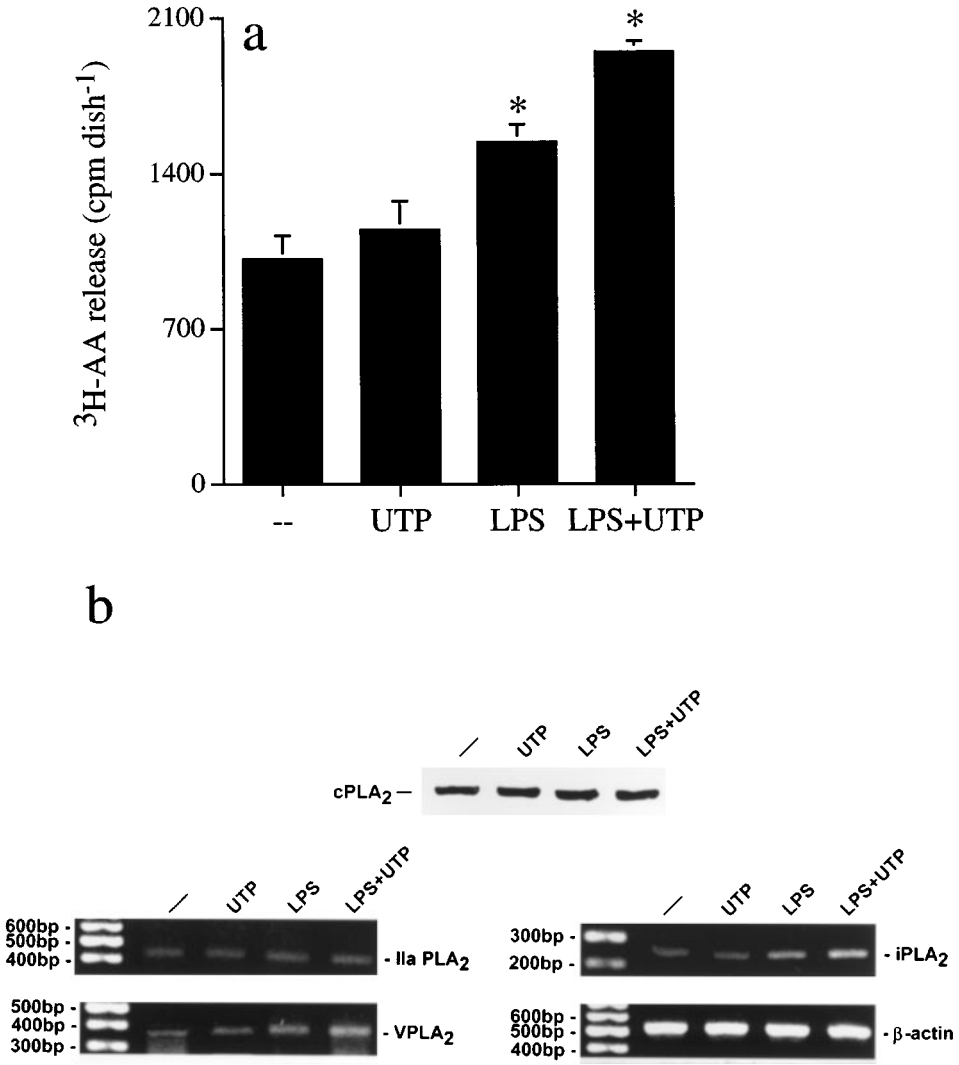

Figure 8 Effects of LPS and UTP on ^{3}H-AA release and PLA_2s expression. (a) Cells were labelled with ^{3}H-AA and treated for 24 h with 1 μg ml^{-1} of LPS either alone or in combination with 100 μM UTP. Basal AA release was measured as described in Methods. The data represent the mean$\pm$s.e.mean of at least three independent experiments performed in duplicate. *$P<0.05$ compared to control ^{3}H-AA release without LPS$\pm$UTP stimulation. (b) mRNA levels of type IIA $sPLA_2$, type V $sPLA_2$ and $iPLA_2$ were quantified by RT–PCR analysis after 8 h treatment with LPS$\pm$UTP and $cPLA_2$ protein levels were quantified by Western blotting after 24 h treatment with LPS$\pm$UTP. The results are representative of three different experiments.

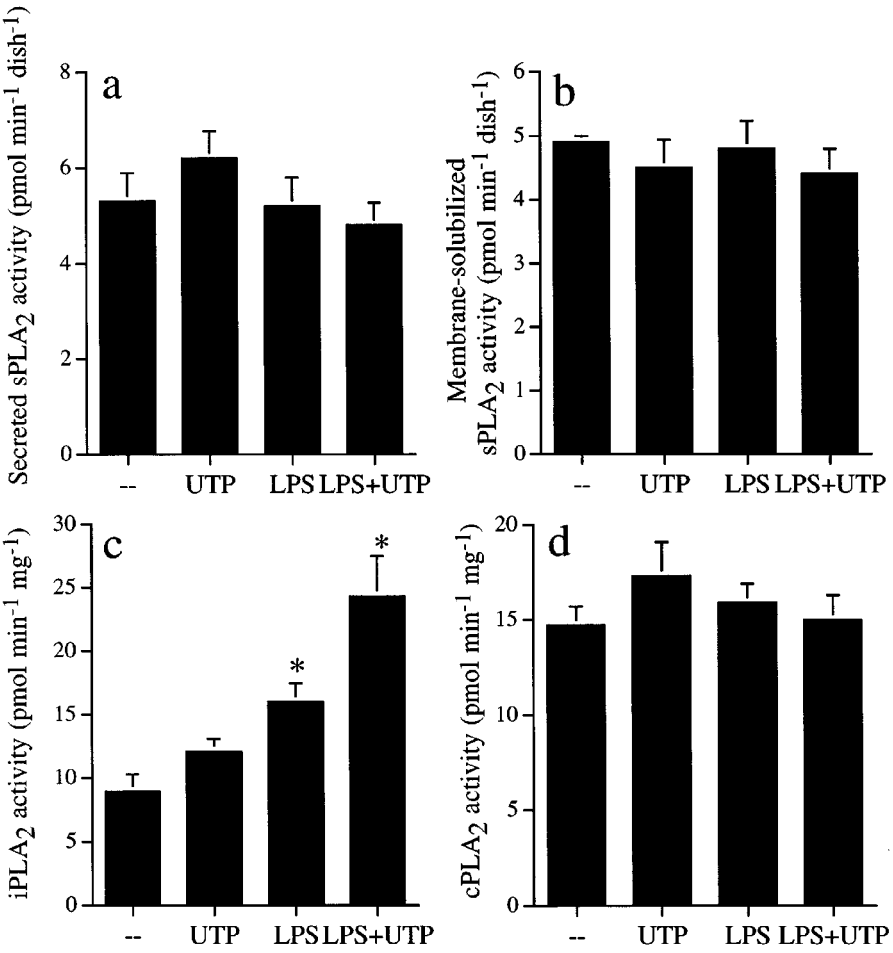

Figure 9 Changes in the activities of $sPLA_2$, $iPLA_2$ and $cPLA_2$ induced by LPS and UTP. Cells were incubated for 24 h with 1 μg ml^{-1} of LPS either alone or plus 100 μM UTP, and the activity of secreted $sPLA_2$ (a), membrane-solubilized $sPLA_2$ (b), $iPLA_2$ (c) and $cPLA_2$ (d) assayed as described in Methods. Values are expressed as the mean$\pm$s.e.mean of 3–4 independent experiments performed in duplicate. *$P<0.05$ compared to the basal activity without LPS$\pm$UTP stimulation.

CaMK and PKC activation. Firstly, of several nucleotide analogues examined within the concentration-range (3–300 μM) previously shown to stimulate PI turnover-elicited downstream signalling in J774 cells (Lin & Chen, 1997; Chen *et al.*, 1998), UTP is the most potent in potentiating LPS-induced PGE_2 release. ATP also caused PGE_2 potentiation, but, at 100 μM, was less effective than UTP. Thus, we believe the action of UTP being mediated by pyrmidinoceptors which we have extensively studied in murine macrophages (Lin & Lee, 1996; Lin & Chen, 1997; Chen *et al.*, 1998; Lin *et al.*, 1998). Supporting this note, our results indicate that $P2Y_6$ receptor is the major P2Y receptor in J774 macrophages. Secondly, the inhibitory effects of U73122 (a PI-PLC inhibitor), BAPTA/AM (an intracellular Ca^{2+} chelator) and KN-93 (a selective CaMK inhibitor) on the UTP-induced PGE_2 potentiation further suggest that the IP_3-mediated $[Ca^{2+}]_i$ increase and the subsequent activation of CaMK are upstream signalling pathways for increasing COX-2 induction. In this context, we have previously detected a $[Ca^{2+}]_i$ increase of approximate 800 nM induced by 100 μM UTP in J774 cells (Lin & Chen, 1997), and we believe that this $[Ca^{2+}]_i$ increase is an absolutely essential determining factor in the downstream signalling leading to COX-2 potentiation (see below). Thirdly, PKC activation by phorbol 12-myristate 13-acetate or over-expression of PKC-ξ isoform has been shown to induce PGE_2 formation in a variety of cells (Baure *et al.*, 1997; Miller *et al.*, 1997). In the present work, both LPS-induced and UTP-potentiated PGE_2 release were inhibited by two PKC inhibitors (Ro 31-8220 and Go 6976), suggesting the involvement of PKCβ activation in COX-2 induction. Consistent with this suggestion, we and others have shown that both LPS and UTP can trigger PKC activation in J774 macrophages (Fujihara *et al.*, 1994; Lin & Chen, 1997). Nevertheless, the greater inhibition of the UTP potentiation effect seen with BAPTA/AM and KN-93 compared with Ro 31-8220 and Go 6976 suggests that Ca^{2+}/CaMK-dependent signalling plays a more important role than PKC activation.

In murine J774 macrophages, our data, as discussed below, confirmed previous findings in other cell types that PGE_2 formation represents a positive feedback pathway in iNOS induction (Koide *et al.*, 1993; Habib *et al.*, 1994). Firstly, nitrite accumulation and iNOS expression induced by LPS alone and potentiated by UTP were inhibited by NS-398 (Figure 5), suggesting that endogenous PGE_2 is not only as a positive regulator on iNOS induction but also, at least in part, as a mediator for UTP-induced iNOS potentiation. Secondly, exogenous PGE_2, at concentrations which was achieved in the medium of UTP-cotreated cells, i.e. 10 pM–10 nM, can also cause a concentration-dependent potentiation of LPS-induced NO accumulation (Figure 7a). Thirdly, the signalling coupled to PGE_2 action in macrophages is known to stimulate adenylyl cyclase and increase cyclic AMP formation, which, in turn, leads to the activation of PKA (Fournier *et al.*, 1995). Here, endogenous PGE_2-triggered cyclic AMP and PKA signal cascades participating in the modulation of iNOS induction

were evidenced by the inhibitory effects of adenylyl cyclase and PKA inhibitors. We found that PKA inhibitor KT5720 was more potent than H-89 in this aspect, the latter cannot significantly reduce NO production until 10 μM. Fourthly, when PGE_2 production was blocked by NS-398, UTP still caused a moderate potentiation of LPS-elicited nitrite accumulation and iNOS expression, suggesting that, in addition to the role played by PGE_2, UTP may act directly on iNOS gene expression as UTP can activate the two transcription factors, NF-κB and AP-1, essential for iNOS gene regulation (Chen *et al*., 1998).

The NF-κB/Rel transcription factors are central to the regulation of proinflammatory immediate-early gene expression, two of them being the iNOS and COX-2 (D'Acqisto *et al*., 1997; Chen *et al*., 1998). It has recently shown that cyclic AMP can activate NF-κB in myeloid cells (Jeon *et al*., 1996) and several mechanisms have been proposed. In this context, PKA-mediated phosphorylation of NF-κB has been shown to be involved in the activation of NF-κB (Blank *et al*., 1992), and this PKA-mediated NF-κB phosphorylation results in an enhancement of its DNA-binding activity *in vitro* (Naumann & Scheidereit, 1994). A further *in vivo* study in *Drosophila schneider* cells (Norris & Manley, 1992) confirmed the positive regulatory role of PKA in nuclear translocation and the transactivating ability of NF-κB. In addition to NF-κB phosphorylation, as demonstrated by Shirakawa & Mizel (1989), PKA can also possibly cause phosphorylation of IκB, thus releasing the active DNA-binding form of NF-κB to translocate to the nucleus to bind κB motifs in the regulatory region of a variety of genes (Shirakawa & Mizel, 1989). In addition to NF-κB, one study in mesangial cells indicated that cyclic AMP can trigger a signalling cascade which activates other nuclear transcription factors, most probably cyclic AMP-responsive element binding protein (CREB), for iNOS gene activation (Eberhardt *et al*., 1994). A mechanism in which cyclic AMP can prolong the half-life of iNOS mRNA has been proposed (Kunz *et al*., 1994). Taken together, these observations imply that complex mechanisms are involved in the stimulation of iNOS expression by the cyclic AMP/PKA system. Our present finding that exogenous PGE_2 is able to stimulate NF-κB implies that the endogenous PGE_2 released following stimulation by LPS plus UTP could maintain the activity of this transcription factor, an essential component of the LPS signalling pathway responsible for iNOS gene activation.

PLA_2 and COX are considered important enzymes in the PGE_2 biosynthetic pathway (Axelrod *et al*., 1988). PLA_2s are enzymes that cleave fatty acids from the sn-2 position of phospholipids. In this study, the effects of LPS and UTP on macrophage PLA_2 activity was also determined, as the possibility exists that PGE_2 production may depend on PLA_2 activity for providing the precursor substrate. Indeed, in agreement with the previous reports that LPS is able to increase PLA_2s (cPLA$_2$ and/or sPLA$_2$) expression in other cells (Arbibe *et al*., 1997; Kuroda *et al*., 1997), we found that basal PLA_2 activity, as assessed indirectly by basal AA release, was increased by 24 h treatment with LPS, and further potentiated by UTP co-addition (Figure 8a). To determine which type of PLA_2 activation provided the substrate for COX-2, we measured cPLA$_2$ immunoreactivity and steady-state mRNA levels of sPLA$_2$ and iPLA$_2$. Our results show that both iPLA$_2$ and type V sPLA$_2$ can be up-regulated by LPS, particularly in combination with UTP. Consistent with the up-regulation of iPLA$_2$ mRNA, we also found that its enzymatic activity was increased. In contrast, we were unable to detect any significant increase in Ca^{2+}-dependent PLA_2 activity in secreted, membrane-solubilized and cytosolic enzyme preparations (Figure 9). Thus, we suggest that iPLA$_2$ plays a major role in the UTP potentiation of LPS-induced AA release.

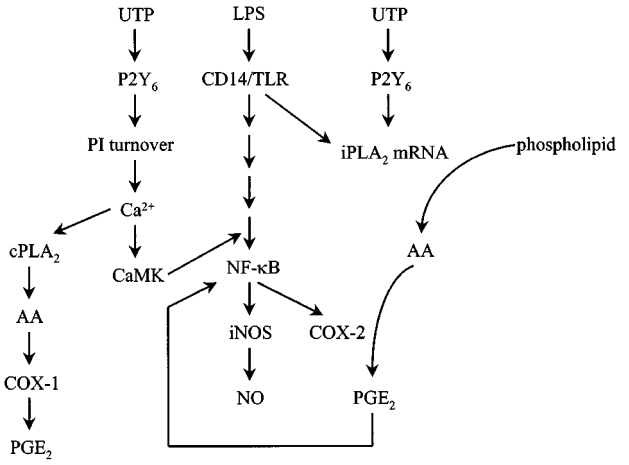

Figure 10 UTP primarily *via* P2Y$_6$ receptor-mediated Ca/CaMK activation potentiates LPS-induced NF-κB activation, which leads to the expression of iNOS and COX-2 genes, and induction of iNOS and COX-2 proteins. UTP can also enhance LPS induction of iPLA$_2$ and release of AA, which provides COX-2 for the formation of PGE_2. The large amount of PGE_2 then positive-feedbackly up-regulates NF-κB-dependent expression of gene iNOS. In the context of AA formation, UTP can also transiently increase Ca^{2+}-dependent cPLA$_2$ and COX-1 activity (Lin & Chen, 1998), while the outcome of PGE_2 release is far less than the amount generated by transcription-dependent COX-2 activation.

Taken together, these results as summarized in Figure 10 for the first time demonstrate the requirement of CaMK-dependent NF-κB activation for UTP potentiation of COX-2 expression, and the contribution of released PGE_2 to the enhancement of iNOS induction in J774 macrophages. This study also highlights UTP as another mediator that can potentiate PGE_2 synthesis *via* increased iPLA$_2$ expression, reinforcing the potential importance of UTP as a regulatory element at inflamed sites.

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