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Oxidized ATP (oATP) attenuates proinflammatory signaling *via* P2 receptor-independent mechanisms

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1 Periodate-oxidized ATP (oATP), which covalently modifies nucleotide-binding proteins, can significantly attenuate proinflammatory signaling. Although the $P2X_7$ nucleotide receptor ($P2X_7R$) is irreversibly antagonized by oATP, it is unclear whether anti-inflammatory actions of oATP are predominantly mediated *via* its actions on $P2X_7R$. Here, we describe inhibitory effects of oATP on proinflammatory responses in three human cell types that lack expression of $P2X_7R$: human umbilical vein endothelial cells (HUVEC), HEK293 cells, and 1321N1 astrocytes.

2 oATP decreased by 40–70% the secretion of interleukin (IL)-8 stimulated by tumor necrosis factor-α (TNF-α) in all three cell types, by IL-1β in HUVEC and 1321N1 cells, and by endotoxin in HUVEC. Attenuation of TNF-α-stimulated IL-8 secretion by oATP was similar in wild-type HEK cells or HEK cells stably expressing recombinant $P2X_7R$.

3 oATP also attenuated cytokine-stimulated expression of nuclear factor-κB-luciferase reporter genes expressed in HEK or 1321N1 cells, but did not affect the rapid downregulation of IκB.

4 oATP had no effect on uridine triphosphate-induced activation of native $P2Y_2$ receptors in HEK cells, but reduced the potency and efficacy of ADP as an agonist of native $P2Y_1$ receptors. However, inhibition of $P2Y_1$ receptors with the specific antagonist MRS2216 did not mimic the effects of oATP on TNF-α-stimulated IL-8 secretion.

5 Although 1321N1 astrocytes lack expression of any known P2 receptor subtypes, oATP markedly inhibited ecto-ATPase activity in these cells, resulting in a significant accumulation of extracellular ATP.

6 In summary, oATP can attenuate proinflammatory signaling by mechanisms independent of the expression or activation of known P2 receptor subtypes.
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Keywords: Purinergic; P2; oATP; oxidized ATP; $P2X_7$ receptor; inflammation; inflammatory signaling; ecto-ATPase; ecto-nucleotidase

Abbreviations: BzATP, 3′-*O*-(4-benzoyl)benzoyl-ATP; HUVEC, human umbilical vein endothelial cells; IL, interleukin; LPS, lipopolysaccharide; 2-meS-ADP, 2-methylthio-ADP; NF-κB, nuclear factor-κB; oATP, periodate-oxidized ATP; $P2X_7R$, $P2X_7$ nucleotide receptor; TNF-αtumor necrosis factor-αUTP, uridine triphosphate; UDP, uridine diphosphate

Introduction

Periodate-oxidized ATP (oATP) was originally developed as an affinity reagent for nucleotide-binding proteins (Lowe & Beechey, 1982; Colman, 1983). Upon prolonged incubation with target proteins, oATP is covalently incorporated *via* a reaction between its dialdehyde group and the ε-amino group on accessible lysine residues (Lowe & Beechey, 1982; Colman, 1983). This has facilitated the use of oATP in modifying and/or labeling ATP-binding sites in a variety of purified proteins, including adenylate cyclase, pyruvate carboxylase, histone kinase, tryptophanyl-tRNA synthetase, sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), and Na^+, K^+-ATPase (Easterbrook-Smith *et al*., 1976; Kochetkov *et al*., 1977; Westcott *et al*., 1980; Fournier *et al*., 1987; Bernikov *et al*., 1990; Mignaco *et al*., 1990).

Murgia *et al*. (1993) subsequently demonstrated that preincubation of intact mouse macrophages with oATP caused a complete and irreversible antagonism of the $P2X_7$ receptor ($P2X_7R$), an ATP-gated ion channel that is predominantly expressed in inflammatory effector cells. This important observation led to the widespread use of oATP in investigations aimed at defining roles for $P2X_7R$ in various signaling pathways that contribute to the overall inflammatory response. Such studies have generally employed oATP to antagonize proinflammatory responses of isolated cells to stimulation by exogenous ATP or 3′-*O*-(4-benzoyl)benzoyl-ATP (BzATP), the most commonly used $P2X_7R$ agonists (Ferrari *et al*., 1997a, b; Lammas *et al*., 1997; Gu *et al*., 1998; Hu *et al*., 1998; Mehta *et al*., 2001). Recently, *in vivo* administration of oATP into inflamed rat paws has been used to investigate the possible role of $P2X_7R$ in perception of inflammatory pain (Dell'Antonio *et al*., 2002). However, oATP treatment has also been shown to attenuate multiple responses to proinflammatory cytokines or endotoxin/lipopolysaccharide (LPS). These include inhibition of LPS-stimulated expression of COX2 (Balboa *et al*., 1999) and iNOS (Hu *et al*.,

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1998) in murine macrophages, attenuation of nuclear factor-κB (NF-κB) activation responses to interleukin (IL)-1β and tumor necrosis factor-α (TNF-α) in primary human astrocytes (Liu *et al*., 2000; John *et al*., 2001), repression of free radical-dependent bacterial killing by LPS- or TNF-α-primed mouse macrophages (Sikora *et al*., 1999), and reduction in LPS-stimulated IL-1β secretion from murine microglial cells (Ferrari *et al*., 1997b). These observations have suggested a model of autocrine regulation wherein LPS or proinflammatory cytokines trigger the release of endogenous ATP, which then acts to stimulate $P2X_7R$ and thereby provide positive feedback to, and amplification of, inflammatory signaling cascades. In this model, oATP antagonizes the autocrine loop of $P2X_7R$ activation and thereby attenuates the net response to the primary proinflammatory mediator. However, direct measurements of ATP release from inflammatory cell types – in amounts sufficient for autocrine activation of the low ATP-affinity $P2X_7R$ – have yielded conflicting results (Ferrari *et al*., 1997a; Sperlágh *et al*., 1998; Grahames *et al*., 1999; Beigi & Dubyak, 2000). Moreover, an inhibitory action of oATP on LPS- and TNF-α-induced NO production was described in macrophages isolated from a $P2X_7R$-knockout mouse line (Sikora *et al*., 1999). These latter observations indicate that the anti-inflammatory actions of oATP may involve P2 receptors other than, or in addition to, $P2X_7R$.

The P2 nucleotide receptors currently include seven P2X family ATP-gated ion channels (North, 2002) and eight P2Y family G-protein-coupled receptors (Abbracchio *et al*., 2003). The latter are variously responsive to ATP and/or other nucleotides including ADP, uridine triphosphate (UTP), uridine diphosphate (UDP), or UDP-glucose. The ability of oATP to attenuate inflammatory responses in primary human astrocytes suggested that P2Y receptor subtypes may be the targets for inhibition, again implicating P2 receptors as potential autocrine regulators of inflammatory signaling (Liu *et al*., 2000; John *et al*., 2001). Such an interpretation is plausible given other reports that the $P2Y_2$ receptor agonist UTP can potentiate inflammatory responses to LPS in murine macrophages (Chen *et al*., 1998), while the UDP-specific $P2Y_6$ receptor can trigger IL-8 production in human monocytes (Warny *et al*., 2001). Nonetheless, the molecular mechanisms that underlie attenuation of inflammatory signaling by oATP remain undefined. No studies have explicitly tested whether oATP might antagonize P2 receptor subtypes, other than $P2X_7R$, that are known to be expressed in inflammatory effector cells.

In this study, we have analyzed the effects of oATP on proinflammatory responses in three human cell types that lack expression of $P2X_7R$: human umbilical vein endothelial cells (HUVEC), HEK293 cells, and 1321N1 astrocytes. Using these various cell models, we demonstrate that oATP can attenuate proinflammatory signaling by mechanisms independent of the expression or activation of P2 receptors.

Methods

Reagents

Firefly-luciferase ATP assay mix (FL-AAM), D-luciferin, potato apyrase (grade I), digitonin, oxidized ATP, ATP, UTP, 2-methylthio-ATP, and suramin were from (Sigma-Aldrich St Louis, MO, U.S.A.). Fura2-AM was from Molecular Probes. MRS2216 (2′-deoxy-2-chloro-N^6-methyladenosine-3′,5′-bisphosphate) was a generous gift from Dr Ken Jacobson (National Institutes of Health, Bethesda, MD, U.S.A.). Recombinant human IL-1β was obtained from the Biological Resources Branch of the NCI-Frederick Cancer Research and Development Center, and recombinant human TNF-α was purchased from R&D Systems. LPS (*Escherichia coli* serotype 0111:B4) was from List Biologicals. The coating antibody (M-801-E), detecting antibody (M-802-B), recombinant human IL-8 standard, and horseradish peroxidase (HRP)-conjugated streptavidin used for the human IL-8 ELISA were from Endogen/Pierce. Primary antibodies against IκB (sc371), p65 NF-κB/RelA (sc109), and COX-2 (sc1745), as well as secondary, HRP-conjugated anti-rabbit or anti-goat antibodies, were from Santa Cruz. The polyclonal anti-$P2X_7$ receptor antibody (APR-004) was from Alamone. pNF-κB-luc and pTAL-luc cDNA expression vectors were from BD-Biosciences/Clontech. Effectene transfection reagent and Qiagen Maxi plasmid isolation kit were from Qiagen. Biocoat collagen-I-coated tissue culture dishes were from Becton Dickinson. Wild-type 1321N1 human astrocytoma cells were a gift from Drs Ken Harden and Jose Boyer (University of North Carolina – Chapel Hill, NC, U.S.A.). Primary HUVECs and endothelial growth medium (EGM) were purchased from Clonetics.

Cell culture

1321N1 human astrocytoma and wild-type HEK293 cells were maintained in Dulbecco's minimal essential medium (DMEM) containing 10% iron-supplemented bovine calf serum (Hyclone), penicillin (100 U ml^{-1}), and streptomycin (100 μg ml^{-1}). Some experiments used HEK293 cells that were stably transfected with the human $P2X_7$ receptor cDNA in the pIRES vector (HEK-$P2X_7$). These HEK-$P2X_7$ cells were subcultured and maintained in DMEM supplemented with 10% calf serum, penicillin (100 U ml^{-1}), streptomycin (100 μg ml^{-1}), and 250 μg ml^{-1} hygromycin as previously described (Gudipaty *et al*., 2003). HUVEC monolayers obtained at passage 1 were cultured on collagen I-coated dishes in EGM media (Clonetics) supplemented with 2% fetal bovine serum, 0.1% human epidermal growth factor (hEGF), 0.1% hydrocortisone, 0.1% GA-1000 (gentamicin sulfate, amphotericin-B), and 0.4% bovine brain extract (BBE). They were passaged weekly by trypsinization and dilution for no more than six serial passages.

Assay for IL-8 induction and release

At 18–24 h prior to experimentation, HUVEC, HEK293 cells, HEK-$P2X_7$ cells, or 1321N1 astrocytes were plated at 5×10^5 cells per well in 24-well plates. HUVEC were plated on collagen I-coated dishes, while HEK cells were plated on poly-D-lysine-coated dishes. On the day of the experiment, the growth medium was removed and the cells incubated with or without oATP (100–600 μM added directly to serum-free DMEM plus 0.1% BSA from sterile stocks) for up to 3 h. In some experiments, the oATP-containing medium was removed and replaced with fresh DMEM plus 10% calf serum prior to inflammatory stimulation of the cells with IL-1β (10 ng ml^{-1}), TNF-α (10 ng ml^{-1}), or LPS (1 μg ml^{-1}). At various times

(1–15 h) following addition of the proinflammatory mediators, samples of the extracellular medium were withdrawn and frozen for subsequent analysis. In other experiments, oATP was additionally included in the cytokine-containing medium used for inflammatory stimulation. Sandwich ELISA protocols were used to assay for IL-8 in the extracellular media samples. Briefly, a 96-well plate was coated with 1 $\mu g\,ml^{-1}$ primary anti-human IL-8 by overnight incubation at room temperature and then blocked with 4% BSA in PBS for 1 h. Plates were washed three times with ELISA buffer (50 mM Tris-HCl, pH 7.5, 0.2% Tween-20). Aliquots (5–25 μl) of media samples diluted to 100 μl with PBS were added to the blocked wells. Other wells were supplemented with known amounts of human IL-8 standard. The plates were incubated at room temperature for 1 h and subsequently washed three times with the ELISA buffer. The washed wells were then supplemented with 100 μl of a secondary, biotinylated anti-human IL-8 antibody diluted to 0.2 $\mu g\,ml^{-1}$ in PBS. The captured immune complexes were further incubated with streptavidin–HRP conjugate (0.1 $\mu g\,ml^{-1}$), washed, and colorimetrically developed with tetramethyl benzidine (TMB) substrate for HRP, and absorbance measurements were taken using a Molecular Devices SoftMax Pro plate reader.

NF-κB-reporter assay

At 18–24 h before transfection, HEK293 cells or 1321N1 astrocytes were plated in DMEM plus 10% calf serum at 2–4 $\times 10^5$ cells per well in 12-well dishes (poly-D-lysine coated for HEK293). The cells were then transfected with 0.3–0.6 $\mu g\,well^{-1}$ of pNF-κB-luc or pTAL-luc cDNA using Effectene (Qiagen) reagent and incubated for 16 h. On the day of the experiment, the transfection medium was removed and the cells incubated with or without oATP (100–600 μM added directly to serum-free DMEM plus 0.1% BSA from sterile stocks) for up to 3 h. In some experiments, the oATP-containing medium was removed and replaced with fresh DMEM plus 10% calf serum prior to inflammatory stimulation of the cells with IL-1β (10 $ng\,ml^{-1}$) or TNF-α (10 $ng\,ml^{-1}$). In other experiments, oATP was additionally included in the cytokine-containing medium used for inflammatory stimulation. After cytokine stimulation, the cells were extracted in lysis buffer containing 25 mM Tris-HCl (pH 7.8), 4 mM EGTA, 15 mM $MgSO_4$, 1% Triton X-100, and 1 mM dithiothreitol. The protein content was determined using the BioRad protein assay kit. Luciferase content was determined by dilution of 50 μl lysate (1 $mg\,ml^{-1}$ protein) into 150 μl FL-AAB assay buffer (Sigma) supplemented with 1.5 mM D-luciferin and 1 μM ATP; luminescence was recorded using a Turner TD20/20 luminometer.

Western blot analyses

Triton X-100 detergent extracted cell lysates were separated *via* SDS–PAGE electrophoresis using 12% gels and then transferred to PVDF membranes for Western blot analysis. IκB was probed with sc371 diluted to 5 $\mu g\,ml^{-1}$; NF-κB-p65/ RelA was probed with sc-515 diluted to 1 $\mu g\,ml^{-1}$; COX2 was probed with sc1745 diluted to 1 $\mu g\,ml^{-1}$; $P2X_7$ receptor protein was probed with Alamone anti-$P2X_7$ antibody diluted to 2 $\mu g\,ml^{-1}$.

Assay of extracellular ATP levels and ecto-ATPase activity

On-line measurements of extracellular ATP concentration, based upon the use of firefly luciferase, were performed using methods first described by Taylor *et al*. (1998) and subsequently modified in this laboratory (Beigi & Dubyak, 2000; Joseph *et al*., 2003). Briefly, 35 mm dishes containing adherent 1321N1 cell monolayers were washed twice with 2 ml of basal saline solution (BSS) containing (in mM): 130 NaCl, 5 KCl, 1.5 $CaCl_2$, 1 $MgCl_2$, 25 Na HEPES (pH 7.5), 5 glucose, and 0.1% BSA. Cell monolayers were allowed to recover in 1 ml BSS for ~45 min at room temperature (22°C) before the start of each assay. A measure of 40 μl of reconstituted FL-AAM was then added to individual 35 mm dishes. ATP-dependent light production was amplified and recorded every 3 min as relative light units (RLU). To assess ecto-ATPase activity, increasing amounts of exogenous ATP were added to cell monolayers. Ecto-ATPase inhibition studies were performed using control 1321N1 monolayers or monolayers that had been preincubated with 300 μM oATP for 3 h prior to washing and assay of extracellular ATP levels. The 10-s integrated RLU recordings of luciferase activity were immediately downloaded into Microsoft Excel using the Turner Designs spreadsheet interface software (version 2.0.1, Sunnyvale, CA, U.S.A.). RLU values were converted to ATP concentrations using calibration curves generated with cell-free samples prepared under identical conditions and tested in parallel with 1321N1 monolayers. Prism 3.0™ software (GraphPad) was used to compute the means and standard deviations of the calculated ATP levels from parallel culture dishes.

Measurement of intracellular Ca^{2+} concentration

Suspensions of trypsin-detached HEK293 or HEK-$P2X_7$ cells were prepared for measurements of intracellular Ca^{2+} concentration by incubation in BSS containing 1 μM fura2 acetoxymethyl ester (fura2-AM) at room temperature for 45 min. The suspensions were washed twice, resuspended at $10^6\,ml^{-1}$ in BSS, and allowed to recover for 10 min at room temperature before fura2 fluorescence (339-nm excitation and 500-nm emission) was measured and calibrated at 37°C in a stirred cuvette as previously described (Gudipaty *et al*., 2003). In some instances, 0.5 $U\,ml^{-1}$ apyrase was added to the suspensions to degrade extracellular ATP and ADP. Pretreatment of cells with oATP was achieved by incubating cells with fresh media containing 300 μM oATP for 3 h before detachment and fura2 loading.

Data evaluation

Data were analyzed and figures were generated using Prism 3.0™ (GraphPad). All experiments were repeated at least two times or as indicated in specific figure legends with similar results.

Results

Effects of oATP on inflammatory activation of human endothelial cells

HUVEC have been widely utilized as primary culture models of endothelial cell function and pathophysiology, including

inflammation (Cines *et al*., 1998). HUVEC express transcripts for $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$ receptors (Jin *et al*., 1998; Wang *et al*., 2002), immunoreactivity for $P2X_4$ and $P2X_6$ receptors (Glass *et al*., 2002), and functional responses to $P2Y_1$- and $P2Y_2$-specific agonists (Ralevic, 2000). Some studies have reported that $P2X_7R$-like apoptotic responses to ATP are also apparent in HUVEC (Goepfert *et al*., 2000). However, the HUVEC used in our experiments did not express detectable levels of immunoreactive $P2X_7R$ in either the basal state or following inflammatory activation by IL-1β (Figure 1b). The presence of $PX2_7R$ in Bacl.2F5 macrophages and COX2 in the IL-1β-treated HUVEC provided positive control signals. These HUVEC cultures also expressed high levels of von Willebrand factor that was secreted in response to acute stimulation with thrombin (data not shown). As a measure of inflammatory activation in HUVEC, we examined the secretion of IL-8 by HUVEC in response to 6-h stimulations with IL-1β, TNF-α, or LPS (Figure 1, open bars). Recent cDNA microarray analyses have indicated that IL-8 is the most strongly upregulated gene product in HUVEC stimulated with proinflammatory mediators (Zhao *et al*., 2001). Pretreatment of HUVEC for 3 h with 300 μM oATP, followed by removal of the oATP prior to stimulation, produced a 40–50% reduction in the amount of IL-8 secreted during the subsequent 6-h stimulations by IL-1β or TNF-α (Figure 1a). A 60–70% attenuation of LPS-induced IL-8 secretion was observed in the oATP-treated cells, but this latter mediator was also the weakest IL-8 secretagogue. These results indicated that cytokine-induced inflammatory signaling in HUVEC can be attenuated by oATP as previously reported for murine macrophages and human astrocytes.

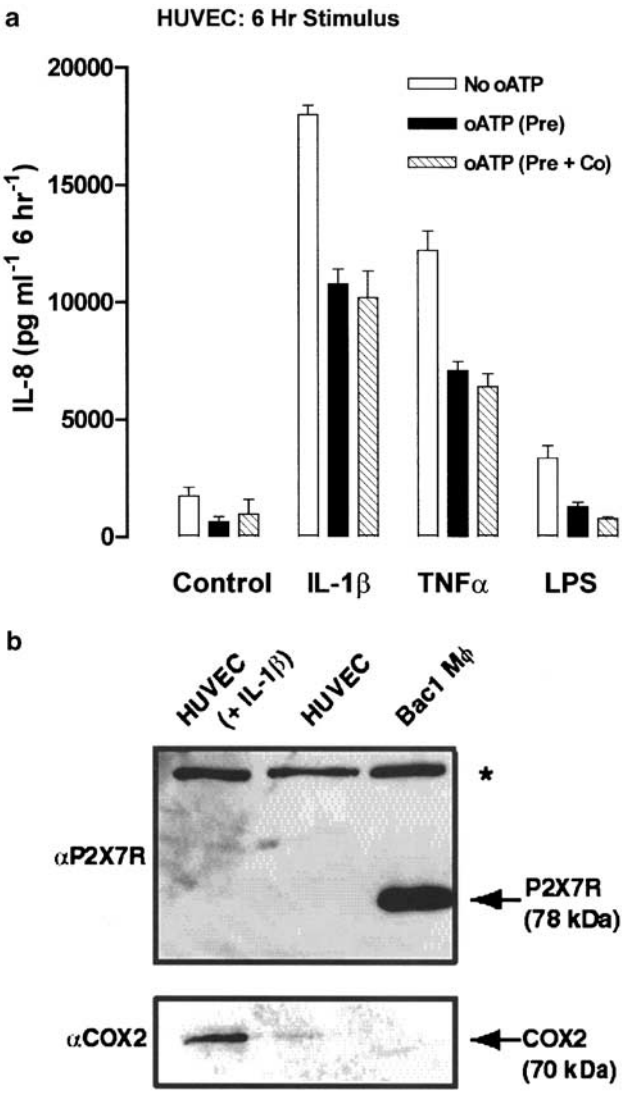


Figure 1 Inhibitory effects of oATP on IL-8 production by HUVEC stimulated with IL-1β, TNF-α, or LPS. (a) HUVEC were incubated with or without 300 μM oATP for 3 h. The oATP-containing medium was then removed and replaced with either fresh control medium (Pre) or fresh oATP-containing medium (Pre + Co). Parallel wells were then variously stimulated with IL-1β (10 ng ml^{-1}), TNF-α (10 ng ml^{-1}), or LPS (1 μg ml^{-1}) for an additional 6 h. Extracellular media samples were then collected and assayed for IL-8 as described in Methods. Data points represent the average $\pm$ range of values from duplicate wells in a single representative experiment. Similar results were observed in two other experiments. (b) Western blot analysis of $P2X_7R$ or COX2 levels in lysates from HUVEC incubated with (left lane) or without 10 ng ml^{-1} IL-1β (*middle lane*) for 15 h. The right lane contains lysate prepared from Bacl.2F5 murine macrophages. *Denotes a nonspecific protein band that reacts with the anti-$P2X_7R$ antibody.

Effects of oATP on inflammatory activation of HEK293 cells

Inhibition of P2 receptor subtypes other than $P2X_7R$ has been proposed to explain the reduced inflammatory responses of oATP-treated human astrocytes (Liu *et al*., 2000) or oATP-treated macrophages from $P2X_7R$-knockout mice (Sikora *et al*., 1999). To address this possibility, we examined inflammatory activation in HEK293 cells. As HEK293 cells natively express $P2Y_1$ and $P2Y_2$ receptors (Schachter *et al*., 1997) but no P2X subtypes, they have been used extensively for functional characterization of recombinant P2X receptors, including $P2X_7$ (Humphreys *et al*., 1998; Kim *et al*., 2001; Gudipaty *et al*., 2003). HEK cells have also been employed to dissect the regulation of proinflammatory NF-κB-based signaling pathways by natively expressed TNF-α receptors (Harper *et al*., 2001) or recombinant Toll-like receptors (Wang *et al*., 2001).

IL-8 synthesis and release was induced in HEK293 cells by stimulation with 10 ng ml^{-1} TNF-α. Significant extracellular accumulation of IL-8 was observed after a 2-h lag phase and continued to increase for the next 3 h (Figure 2a). Pretreatment of the HEK cells with oATP for 3 h produced 35–50% decreases in the rate and extent of IL-8 secretion. Consistent with previous studies (Hu *et al*., 1998; Balboa *et al*., 1999; Liu *et al*., 2000), the maximally effective concentration of oATP as an anti-inflammatory agent was in the 100–300 μM range. As with HUVEC, the continued presence of oATP during the subsequent stimulation of HEK cells with TNF-α further attenuated the extent of IL-8 secretion (Figure 2c). To test whether the additional expression of $P2X_7R$, a known oATP target, altered these patterns of TNF-α-induced IL-8 secretion, we performed similar studies using a previously described HEK293 cell line that stably expresses the human $P2X_7R$ (Humphreys *et al*., 1998; Gudipaty *et al*., 2003). The presence of $P2X_7R$ at high copy number (Figure 2b) did not significantly enhance the extent of TNF-α-induced IL-8 release nor did it alter the relative inhibitory actions of oATP (Figure 2d).

As an alternative index of inflammatory activation, we assayed TNF-α-stimulated induction of a transiently transfected NF-κB luciferase reporter construct in HEK293 cells, with or without oATP treatment. Figure 3a illustrates the results of a representative experiment performed in triplicate, while Figure 3b shows the averaged results from four separate experiments. The induction of NF-κB, stimulated by TNF-α, was reduced by 35% in cells pretreated with oATP. The continued presence of oATP during the TNF-α induction period further increased the level of inhibition to >70%. In other experiments, we verified that TNF-α *per se* and TNF-α plus oATP did not alter expression of a constitutively expressed luciferase reporter (pTAL) transiently transfected into HEK293 cells (data not shown).

Given this inhibitory action of oATP on the TNF-α-dependent induction of an NF-κB reporter gene, we tested whether oATP might also affect an early signaling reaction in the TNF-α receptor→NF-κB activation cascade: the rapid degradation of the inhibitory IκB subunit subsequent to its phosphorylation by Iκ-kinases. Figure 3c shows that TNF-α-induced degradation of IκB in HEK cells was nearly complete within 10 min and that oATP pretreatment had no effect on the rate or extent of this degradation. Likewise, oATP pretreatment did not markedly alter the cellular levels of the p65-NF-κB/RelA subunit. Thus, the inhibitory effect of oATP on expression of NF-κB-regulated genes does not involve repression of the proximal kinase cascades activated by proinflammatory cytokine receptors.

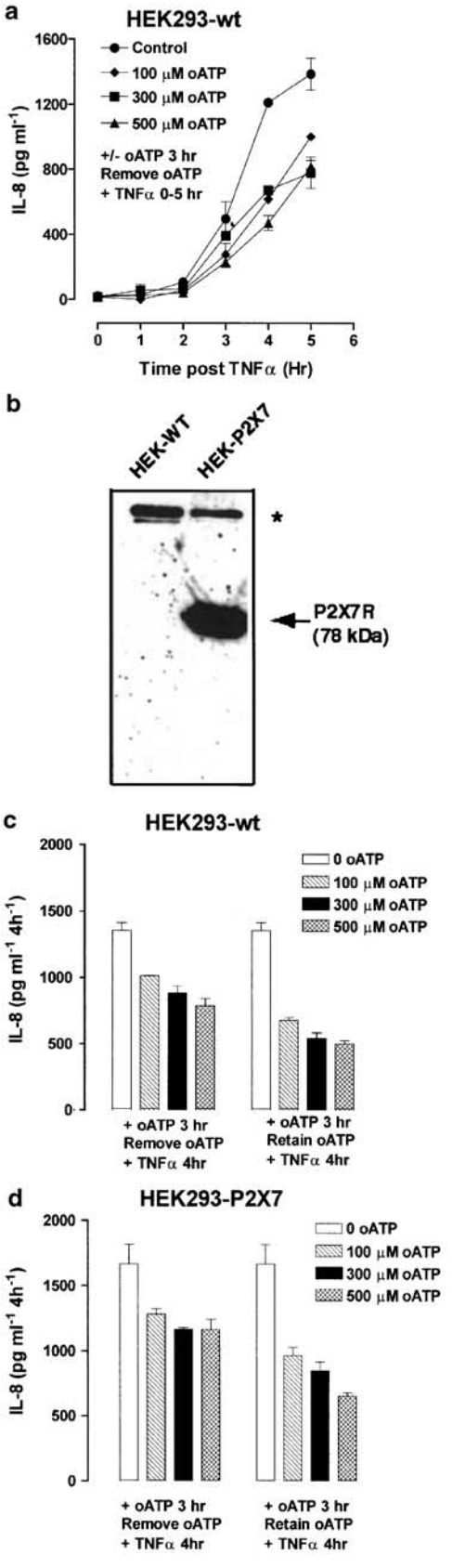


Figure 2 Inhibitory effects of oATP on IL-8 production by HEK293 cells stimulated with TNF-α. (a) Wild-type HEK293 cells (HEK293-wt) were incubated with or without the indicated concentrations of oATP for 3 h. The oATP-containing medium was then removed and replaced with fresh control medium. The cells were then stimulated with or without 10 ng ml^{-1} TNF-α. At the indicated times, extracellular media samples were then collected and assayed for IL-8. Data points represent the average ± range of values from duplicate wells in a single representative experiment. A similar time course was observed in one other experiment. (b) Western blot analysis of $P2X_7R$ levels in lysates from wild-type HEK293 cells (HEK-wt) or HEK293 cells stably transfected with cDNA encoding human $P2X_7R$ (HEK-$P2X_7$). *Denotes a nonspecific protein band that reacts with the anti-$P2X_7R$ antibody. (c) Wild-type HEK293 cells were incubated with or without the indicated concentration of oATP for 3 h. The oATP-containing medium was then removed and replaced with either fresh control medium (Pre) or fresh oATP-containing medium (Pre + Co). Parallel wells were then stimulated with TNF-α (10 ng ml^{-1}) for an additional 4 h. Extracellular media samples were collected and assayed for IL-8 as described in Methods. Data points represent the average ± range of values from duplicate wells in a single representative experiment. Similar results were observed in one other experiment. (d) Similar experiment as in panel c but using the HEK-$P2X_7$ cell line. Data points represent the average ± range of values from duplicate wells in a single representative experiment.

Effects of oATP on the native $P2Y_1$ and $P2Y_2$ receptors of HEK293 cells

In the absence of $P2X_7R$, the natively expressed $P2Y_1$ and $P2Y_2$ receptors of HEK cells comprise an alternative set of nucleotide-binding proteins that might be modified by extracellular oATP. As an index of $P2Y_1R$ or $P2Y_2R$ activation, we assayed Ca^{2+}-mobilization responses to 2-methylthio-ADP (2-meS-ADP) and ADP as $P2Y_1$-selective agonists or to UTP as a $P2Y_2$-selective agonist. For most of these experiments, we used the HEK line that stably expresses recombinant human $P2X_7R$ because this latter receptor provided an internal positive

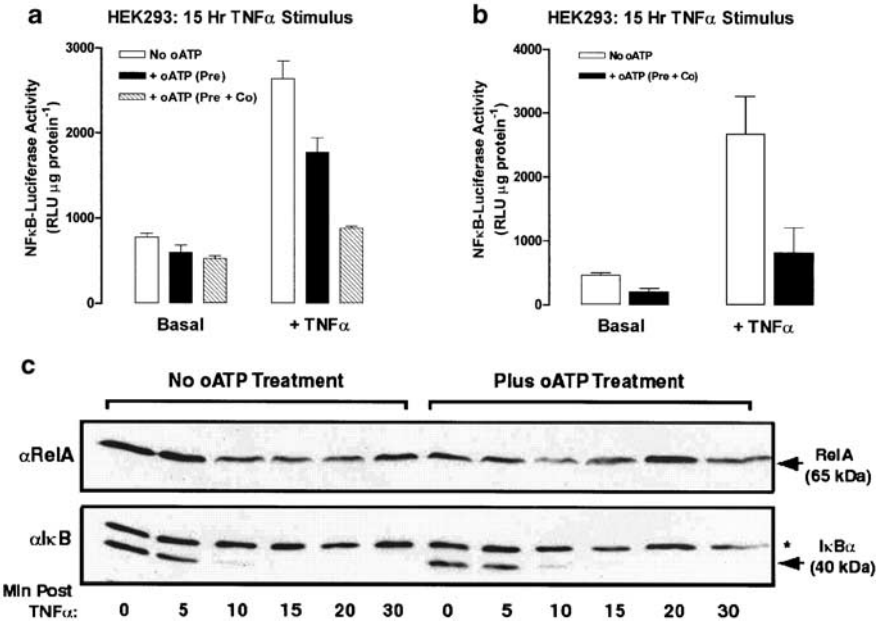


Figure 3 Effects of oATP treatment on TNF-α-induced activation of NF-κB signaling and NF-κB reporter expression in HEK293 cells. (a) Wild-type HEK293 cells were transiently transfected with pNF-κB-luc expression vector as described in Methods. At 16 h post-transfection, the cells were transferred to fresh medium with or without 300 μM oATP and incubated for 3 h. The oATP-containing medium was then removed and replaced with either fresh control medium (Pre) or fresh oATP-containing medium (Pre + Co). The cells were then stimulated with TNF-α (10 ng ml^{-1}) for an additional 15 h prior to cell lysis and analysis of luciferase expression. Data points represent the mean $\pm$ s.d. of values from triplicate wells in a single representative experiment. (b) Experiments were performed as described in panel a. Data points represent the mean $\pm$ s.e. of values from four separate experiments. (c) Parallel wells of adherent wild-type HEK293 cells were incubated with or without 300 μM oATP for 3 h. The oATP-containing medium was then removed and replaced with fresh medium. The cells were then stimulated with 10 ng ml^{-1} TNF-α for the indicated times prior to lysis, SDS–PAGE, and Western blot analysis of the cellular levels of the p65-NF-κB subunit (Rel A) or the IκBα subunit. These blots are representative of results from two separate experiments. *Denotes a nonspecific protein band that reacts with the anti-IκB antibody.

control of oATP's antagonistic efficacy. However, identical results were observed using wild-type HEK293 cells. Figure 4a shows the transient Ca^{2+}-mobilization responses to maximally activating concentrations (30 μM) of 2-meS-ADP and UTP followed by the massive and sustained Ca^{2+} influx triggered by the activation of $P2X_7R$ with 3 mM ATP. Although the activation of $P2X_7R$ was completely inhibited by oATP pretreatment, the $P2Y_1$ and $P2Y_2$ receptors remained functional (Figure 4b).

The peak magnitude of the Ca^{2+}-mobilization response to 2-meS-ADP was reduced in oATP-treated cells. One possible reason for this reduction in $P2Y_1R$ responsiveness was partial desensitization of the receptor due to altered regulation of extracellular ATP/ADP accumulation and catabolism. To address this possibility, Ca^{2+}-mobilization responses were measured in the presence of potato apyrase (Plesner, 1995). The presence of apyrase in the extracellular medium ensured that basal concentrations of ATP and ADP were maintained below the activation thresholds of $P2Y_1R$ or $P2Y_2R$, thereby maintaining the receptors in a sensitized condition. As illustrated in Figure 4c, inclusion of apyrase in the assay medium did not interfere with activation of $P2Y_1$, $P2Y_2$, or $P2X_7$ receptors. In the presence of apyrase, the oATP-treated cells continued to exhibit the complete attenuation of $P2X_7R$ activation, no effect on $P2Y_2R$ signaling, and a modest decrease in the peak Ca^{2+} mobilization triggered by $P2Y_1R$ (Figure 1d). Under these conditions, the transient increase in Ca^{2+} triggered by 3 mM ATP reflected stimulation of $P2Y_2$ receptors that became resensitized for activation following the rapid apyrase-mediated degradation of the previously added 30 μM UTP.

To examine the potential effects of oATP on $P2Y_1R$ and $P2Y_2R$ activation in more detail, concentration–response relationships were determined by plotting normalized peak Ca^{2+} mobilization as a function of ADP or UTP concentrations in control *versus* oATP-pretreated HEK cells. Consistent with the previous findings of Murgia *et al*. (1993), we observed no effect of oATP on the potency or efficacy of UTP as an activator of $P2Y_2R$ (data not shown). In contrast, pretreatment with oATP produced a partial antagonism of $P2Y_1R$ activation (Figure 5). This antagonism was characterized by reductions in both the potency (EC_{50} increased from 0.7 to 4.7 μM) and efficacy (50% decrease in peak Ca^{2+}) of ADP as a $P2Y_1R$ agonist. The reduction in ADP efficacy suggests a decrease in the absolute number of cell surface $P2Y_1R$ that are available for activation.

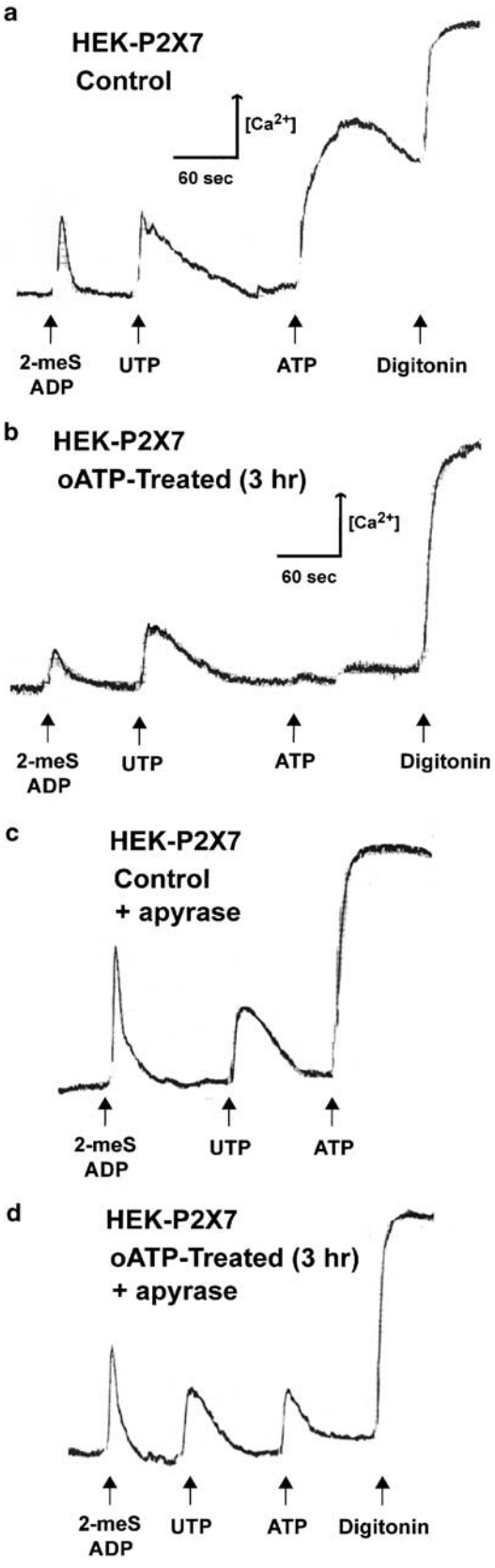
Effects of other P2Y$_1$ R antagonists on inflammatory activation of HEK293 cells

That oATP treatment partially antagonized ADP activation of $P2Y_1R$ suggested that $P2Y_1R$ could be a target of oATP that mediates its anti-inflammatory actions in HEK cells. This would also imply that autocrine activation of $P2Y_1R$ by released pools of endogenous ATP/ADP may act to reinforce the net inflammatory response to TNF-α. If so, other $P2Y_1R$

antagonists should mimic the inhibitory actions of oATP on TNF-α-induced responses. We tested this possibility by examining the actions of MRS2216 (2′-deoxy-2-chloro-N^6-methyladenosine-3′,5′-bisphosphate) and suramin on TNF-α induction of IL-8 expression and secretion (Figure 6). MRS2216 belongs to a series of selective and high-affinity $P2Y_1R$ antagonists synthesized by Jacobson and co-workers (Nandanan *et al*., 1999; Jacobson *et al*., 2002). We verified that 1 μM MRS2216 completely antagonized ADP-induced Ca^{2+} mobilization in HEK cells, while having no effect on either UTP-elicited Ca^{2+} transients or the Ca^{2+} influx triggered by $P2X_7R$ activation (data not shown). However, at concentrations as high as 30 μM, MRS2216 had no inhibitory effect on TNF-α-stimulated production of IL-8.

Suramin has been widely used as a nonselective antagonist of multiple P2 receptor subtypes with reported IC_{50} values for $P2Y_1R$ in the 1–10 μM range (Jacobson *et al*., 2002). However, 100 μM suramin had no effect on the IL-8 secretion response to TNF-α (Figure 6). At 300 μM, suramin produced a 30% attenuation of IL-8 release, but this likely represents a direct inhibitory effect on the binding of TNF-α to its receptor (Grazioli *et al*., 1992).

The inability of these $P2Y_1R$ antagonists to repress proinflammatory responses to TNF-α in HEK293 cells indicated that the partial antagonism of $P2Y_1R$ function by



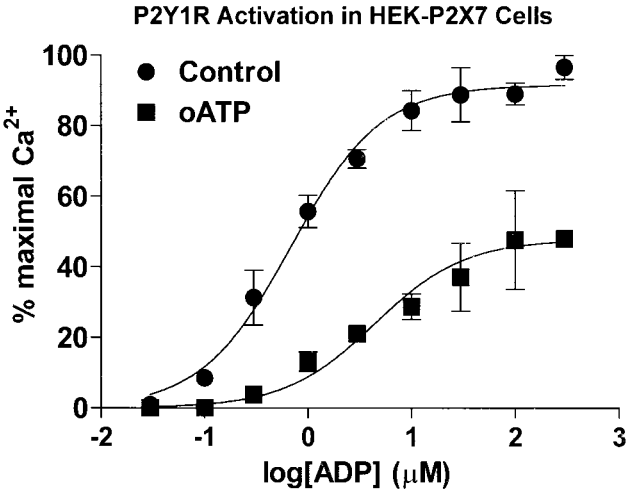


Figure 5 Concentration–response relationships for ADP stimulation of $P2Y_1R$-mediated Ca^{2+} responses in control *versus* oATP-treated HEK-$P2X_7$ cells. Control or oATP-preincubated HEK-$P2X_7$ cells were prepared as described in Methods and resuspended in BSS containing 0.5 U ml^{-1} apyrase. Changes in cytosolic Ca^{2+} were recorded immediately after exposing cells once to the indicated concentrations of ADP. Peak fluorescence (nM Ca^{2+}) was determined for each transient and normalized ('% peak Ca^{2+}') relative to the maximal response for the entire cell preparation. Indicated values are mean ± s.e. for $n=4$ (control) or $n=3$ (oATP) separate experiments. Sigmoidal dose–response curves were fit to each set of data from which EC_{50} values were extracted.

Figure 4 Effects of oATP on intracellular calcium transients stimulated by agonists for $P2Y_1$ receptors, $P2Y_2$ receptors, or $P2X_7$ receptors in HEK-$P2X_7$ cell suspensions. HEK-$P2X_7$ cells were loaded with fura-2 as described in Methods and equilibrated to 37°C in a stirred cuvette before being stimulated with maximally activating concentrations of 2-meS-ADP (30 μM), UTP (30 μM), or ATP (3 mM). Cells were permeabilized with 20 μg ml^{-1} digitonin at the conclusion of each experiment. (a) Control cells. (b) Cells pretreated with 300 μM oATP for 3 h prior to fura2 loading. (c and d) Cells prepared identically to those in (a and b), respectively, with the inclusion of 0.5 U ml^{-1} apyrase in final resuspension buffer to maintain P2 receptors in a sensitized condition. These traces are representative of results from three to four similar experiments.

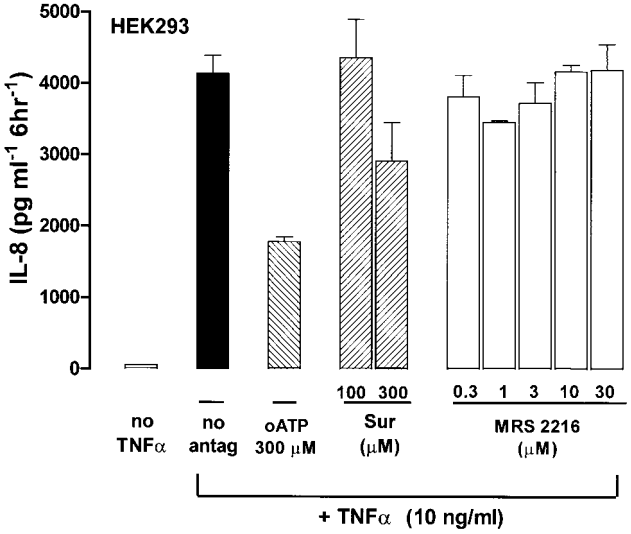


Figure 6 Comparative inhibitory effects of oATP, suramin, and MRS2216 on IL-8 production by HEK293 cells stimulated with TNF-α. HEK293 cells were incubated with or without 300 μM oATP for 3 h. The medium from the oATP-treated cells was then removed, replaced with fresh oATP-containing medium, and the cells were stimulated with 10 ng ml^{-1} TNF-α for 6 h. Other wells of non-oATP-treated cells were pretreated with the indicated concentrations of suramin or MRS2216 prior to stimulation with TNF-α. Extracellular media samples were then collected and assayed for IL-8. Data points represent the average ± range of values from duplicate wells in a single representative experiment. Similar results were observed in two other experiments.

oATP is an unlikely explanation for its anti-inflammatory actions. Coupled with the absence of any effect on $P2Y_2R$, the only other P2 receptor natively expressed in these HEK cells, these observations suggested that oATP can attenuate inflammatory activation by targeting signaling pathways that are independent of P2 receptor expression or function.

Effects of oATP on inflammatory activation of 1321N1 astrocytes

To determine whether oATP might modulate inflammatory responses by P2 receptor-independent mechanisms, we tested the actions of oATP on the 1321N1 human astrocytoma cell line. These cells lack expression of the known P2Y or P2X receptors and have proven very useful as a P2-null background for the heterologous expression and functional characterization of various Gq-coupled P2Y receptor subtypes (Parr *et al*., 1994; Schachter *et al*., 1996), the Gs/Gq-coupled $P2Y_{11}$ receptor (Communi *et al*., 1999), the Gi-coupled $P2Y_{13}$ receptor (Communi *et al*., 2001), and several P2X receptor subtypes (Bianchi *et al*., 1999). Although the presence of the Gi-coupled $P2Y_{12}$ receptor has been described in primary astrocytes (Hollopeter *et al*., 2001), Western blot analysis of our 1321N1 astrocytoma line with a selective anti-$P2Y_{12}$ antibody revealed no detectable expression of this ADP-specific receptor (P.B. Conley, personal communication).

Similar to primary astrocytes, 1321N1 cells do express the requisite signaling machinery for regulation of NF-κB-based signaling pathways by TNF-α (Hernandez *et al*., 2001), IL-1β (Moynagh *et al*., 1994), or recombinant $P2Y_6$ receptors (Warny *et al*., 2001). The ability of IL-1β (Figure 7a) or TNF-α (Figure 7b) to activate expression of an NF-κB-regulated luciferase reporter was significantly reduced (by 50–70%) following a 2-h preincubation of 1321N1 cells with 300 μM oATP. We also compared endogenous IL-8 production by control or oATP-treated 1321N1 cells during 6-h stimulations with IL-1β or TNF-α. Cells that were pretreated with oATP for 2.5 h and then washed prior to the cytokine exposure were characterized by a modest reduction in IL-8 release with a 30% inhibition in the response to IL-1β *versus* a 20% reduction in the response to TNF-α (Figure 8a). In contrast to HUVEC, 1321N1 cells were unresponsive to LPS. When the added oATP was maintained throughout the 6-h stimulation with cytokines, the antagonistic efficacy was increased to produce 40 or 50% reductions in the respective responses to IL-1β or TNF-α. Similar to our observations with HEK293 fibroblasts, the ability of proinflammatory cytokines to induce IL-8 expression in 1321N1 astrocytoma cells was also markedly attenuated by high (≥100 μM) concentrations of the nonselective P2 receptor antagonist suramin, but not by the $P2Y_1$ receptor-specific antagonist MRS2216 (Figure 8b).

Effects of oATP on ecto-ATPase activity and accumulation of extracellular ATP

As part of their initial description of the antagonistic actions of oATP on $P2X_7R$ function, Murgia *et al*. (1993) reported that oATP-treated J774 murine macrophages also exhibited a reduction in ecto-ATPase activity. We have previously described an on-line, luciferase-based system that permits continuous assay of extracellular ATP levels in monolayers of various cell types including adherent macrophages (Beigi & Dubyak, 2000) and 1321N1 astrocytes (Joseph *et al*., 2003). Using this assay, we examined rates of ATP hydrolysis and basal accumulation of extracellular ATP in 1321N1 cell cultures with and without oATP pretreatment (Figure 9). Basal, steady-state extracellular ATP concentrations were elevated 90-fold for oATP-treated 1321N1 astrocytes (~90 *versus* ~1 nM for control cells), indicating that they were unable to clear the ATP released from endogenous stores due to the mechanical stress from washing and addition of fresh assay medium (Beigi & Dubyak, 2000; Lazarowski *et al*., 2000; Joseph *et al*., 2003). Consistent with this finding, cells pretreated with oATP exhibited a much reduced capacity (>90% inhibition) to catabolize pulses of exogenously added ATP. Similar effects of oATP pretreatment on steady-state ATP accumulation were observed in several other cell types, including BAC1.2F5 and RAW 264.7 murine macrophages and RBL-2H3 rat basophilic leukemia cells (data not shown). Thus, oATP preincubation of intact cells causes a profound dysregulation of extracellular nucleotide metabolism that remains in effect for at least several hours after removal of the oATP.

Discussion

The studies described in this report confirm and extend the observations of Sikora *et al*. (1999) that oATP can attenuate proinflammatory signaling by $P2X_7R$-independent mechanisms. Such inhibitory effects of oATP were observed in three different human cell types devoid of $P2X_7R$ expression: HUVEC, HEK293 cells, and 1321N1 astrocytes (Figures 1–3, 6–8). Furthermore, this inhibitory effect was not increased in HEK293 cells stably expressing the human $P2X_7R$

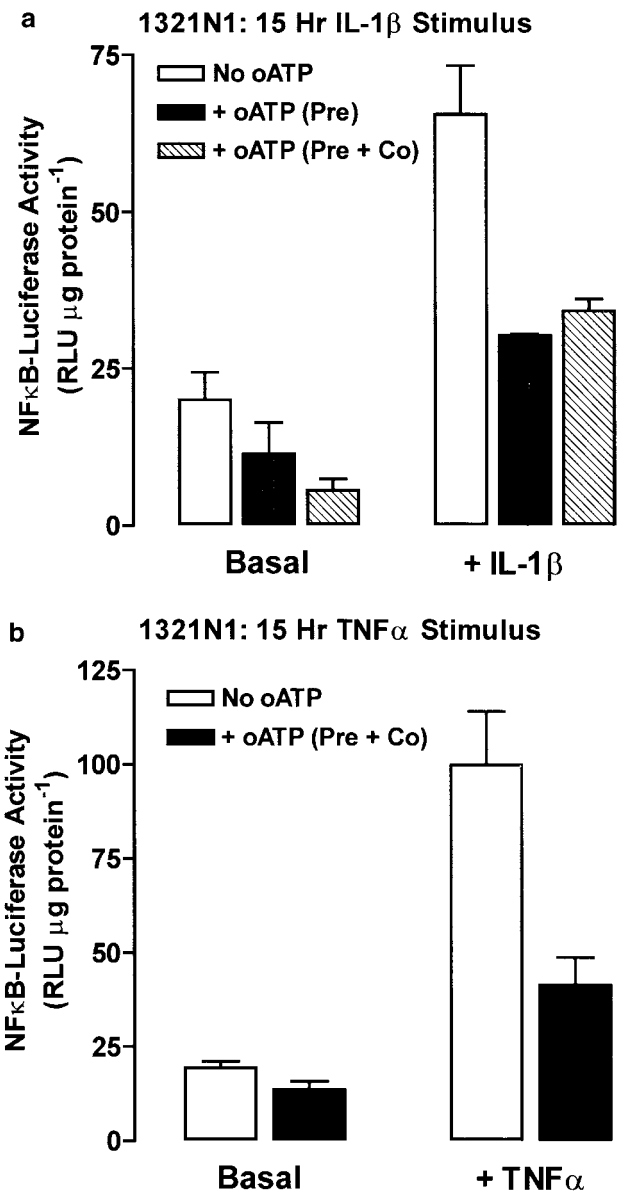


Figure 7 Effects of oATP treatment on IL-1β- or TNF-α-induced activation of NF-κB reporter expression in 1321N1 astrocytes. (a) 1321N1 astrocytes were transiently transfected with pNF-κB-luc expression vector as described in Methods. At 16 h post-transfection, the cells were transferred to fresh medium with or without 300 μM oATP and incubated for 2 h. The oATP-containing medium was then removed and replaced with either fresh control medium (Pre) or fresh oATP-containing medium (Pre + Co). The cells were then stimulated with IL-1β (10 ng ml^{-1}) for an additional 15 h prior to cell lysis and analysis of luciferase expression. Data points represent the mean $\pm$ s.d. of values from six separate experiments. (b) Experiments were performed as described in panel a but using TNF-α (10 ng ml^{-1}) as the stimulus. Data points represent the mean $\pm$ s.e. of values from triplicate wells in a single experiment.

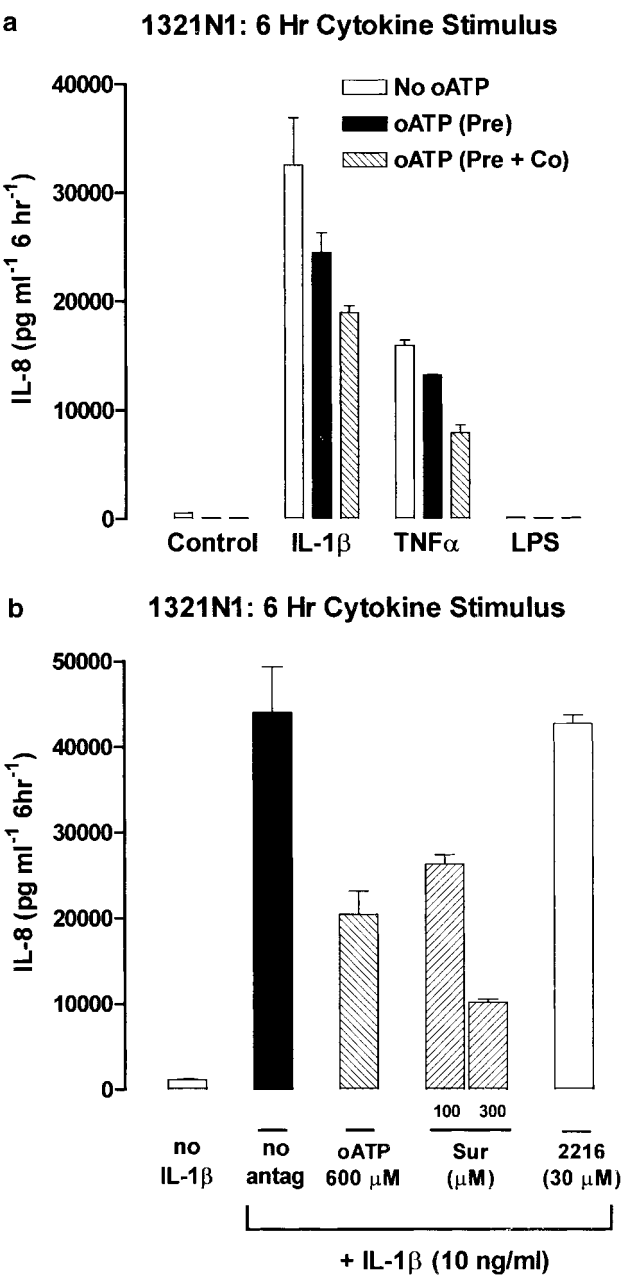


Figure 8 Comparative inhibitory effects of oATP, suramin, and MRS2216 on IL-8 production by 1321N1 astrocytes stimulated with proinflammatory mediators. (a) 1321N1 astrocytes were incubated with or without 300 μM oATP for 2.5 h. The oATP-containing medium was then removed and replaced with either fresh control medium (Pre) or fresh oATP-containing medium (Pre + Co). Parallel wells were then variously stimulated with IL-1β (10 ng ml^{-1}), TNF-α (10 ng ml^{-1}), or LPS (1 μg ml^{-1}) for an additional 6 h. Extracellular media samples were collected and assayed for IL-8. Data points represent the average $\pm$ range of values from duplicate wells in a single representative experiment. Similar results were observed in two other experiments. (b) 1321N1 astrocytes were incubated with or without 600 μM oATP for 3 h. The medium from the oATP-treated cells was then removed, replaced with fresh oATP-containing medium, and the cells were stimulated with 10 ng ml^{-1} IL-1β for 6 h. Parallel wells of non-oATP-treated cells were pretreated with indicated concentrations of suramin or MRS2216 prior to stimulation with IL-1β. Extracellular media samples were then collected and assayed for IL-8. Data points represent the average $\pm$ range of values from duplicate wells in a single representative experiment. Similar results were observed in one other experiment.

(Figure 2). More significantly, the finding that oATP potently reduced inflammatory signaling in 1321N1 astrocytes, a cell type known to lack expression of all known P2 receptor subtypes (Figures 7 and 8), indicated that the anti-inflammatory actions of oATP were mediated in large part by a mechanism(s) independent of the expression or activation of any P2 receptors.

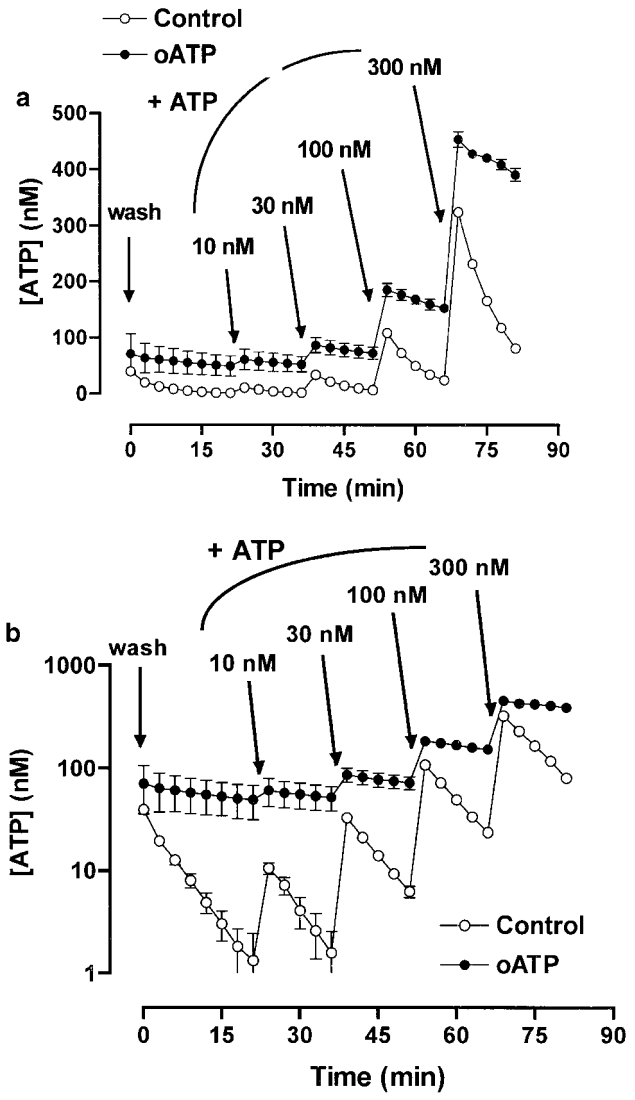


Figure 9 Preincubation of 1321N1 astrocytes with oATP inhibits cellular ecto-ATPase activity and significantly elevates steady-state extracellular ATP levels. 1321N1 astrocytes were grown and prepared for on-line extracellular ATP assays in 35-mm dishes as described in Methods. All traces represent the mean$\pm$s.d. of triplicate samples grown and tested in parallel. (a) Extracellular ATP concentrations plotted on a linear scale. (b) The same data as in (a), plotted on a logarithmic scale to emphasize differences in exponential ATP decay rates between control and oATP-pretreated cells.

It should be stressed that the anti-inflammatory effects of oATP observed in some systems undoubtedly include antagonism of $P2X_7R$-based signaling. Likewise, our new observation demonstrating a partial downregulation of $P2Y_1R$ responsiveness in oATP-treated HEK293 cells (Figure 5) supports the hypothesis that modulation of $P2Y_1R$, and perhaps other P2Y subtypes, may also contribute to the anti-inflammatory actions of oATP observed in certain cell types (Liu *et al*., 2000; John *et al*., 2001). It was observed that MRS2216, a specific $P2Y_1R$ antagonist, failed to have any pronounced effect upon inflammatory signaling in HEK293 and 1321N1 cells (Figures 6 and 8). Nonetheless, the inhibitory efficacy of oATP on IL-1β- and TNF-α-induced IL-8 secretion was more pronounced in HUVEC, which express multiple P2 receptor subtypes, than in 1321N1 astrocytes, which express no P2 receptors (compare Figures 1a and 8a). Taken together, these findings indicate that oATP cannot be used as a mechanistically informative reagent for defining the autocrine/paracrine roles of particular P2 nucleotide receptor subtypes in complex inflammatory responses.

Given the widespread use of oATP as a $P2X_7R$ antagonist, it is relevant to consider the conditions under which oATP can be used as an unambiguous probe of $P2X_7R$ function. Previous studies have established (and this study has confirmed) the ability cf oATP to completely and irreversibly block the immediate ionotropic responses (e.g., Ca^{2+} influx and depolarizing inward current) to $P2X_7$ receptor activation by exogeneous ATP or BzATP, while largely sparing the Ca^{2+}-mobilization responses to ADP/ATP-sensitive $P2Y_1$ and $P2Y_2$ receptors. However, acute exposure to oATP (100–300 μM) has been shown to repress partially (60%) the inward currents elicited by $P2X_1$ or $P2X_2$ receptors expressed in HEK293 cells (Evans *et al*., 1995), Prolonged oATP pretreatment can repress the ionotropic actions of $P2X_2$ homomeric or $P2X_2/P2X_3$ heteromeric channels by up to 85% (A. Suprenant, personal communication). Certain cell types, such as rat parotid acinar cells (Tenneti *et al*., 1998) and rat microglial cells (Visentin *et al*., 1999), natively coexpress $P2X_7$ together with $P2X_4$ receptors. However, Visentin *et al*. (1999) observed that oATP treatment of rat microglial cells completely repressed $P2X_7R$-dependent currents while producing only a 24% reduction in an ATP-gated channel with a $P2X_4$-like pharmacology. Thus, oATP-dependent ablation of ATP-induced changes in inward current or Ca^{2+} influx in most cell types will predominantly reflect antagonism of P2X7R.

The mechanism(s) by which oATP exerts its P2 receptor-independent actions on proinflammatory signaling remains to be defined. It is worth noting that two other nonspecific P2 receptor antagonists, suramin and PPADS (pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid), have also been shown to interfere with inflammatory signaling in macrophages and astrocytes (Lambrecht *et al*., 1992; Hu *et al*., 1998; Balboa *et al*., 1999; Sikora *et al*., 1999; Liu *et al*., 2000; John *et al*., 2001). We observed that suramin significantly reduced the cytokine-induced expression of IL-8 in HEK293 fibroblasts and 1321N1 astrocytes (Figures 6 and 8). However, the ability of suramin to attenuate inflammatory signaling by IL-1, IL-2, IL-4, and TNF-α in other cell types has been shown to reflect disruption of the binding interaction between these cytokines and their cognate receptors (Mills *et al*., 1990; Grazioli *et al*., 1992; Strassmann *et al*., 1994; Leland *et al*., 1995). Suramin also induces shedding of the hepatocyte growth factor receptor in several cell lines (Galvani *et al*., 1995). Thus, there is precedent for the possibility that oATP might interfere directly with the activation of cytokine receptors or the growing family of mammalian Toll-like receptors (Means *et al*., 2000).

Alternatively, oATP may enter cells and thereby attenuate inflammatory signaling downstream from cytokine receptors *per se*. It is conceivable that this compound may gain access to cytoplasmic spaces *via* fluid-phase pinocytosis because prolonged incubation (>60 min) with submillimolar concentrations of oATP is required for maximal antagonism of $P2X_7R$ (Murgia *et al*., 1993). The ability of oATP to attenuate NF-κB-dependent gene expression in intact cells, as well as its ability to modify and inhibit various purified kinases covalently, raised an obvious question as to whether internalized oATP might target the IκB-kinase complex that facilitates the

dissociation of cytosolic p50/p65/IκB complexes, rapid degradation of IκB, and consequent nuclear accumulation of transcriptionally active NF-κB subunits (Ghosh & Karin, 2002). However, we observed no differences in the rate or extent of IκB degradation triggered by TNF-α in control *versus* oATP-treated HEK293 cells. This suggests that oATP treatment does not affect the nuclear accumulation of NF-κB, but rather retards the ongoing process of transcribing NF-κB-regulated genes with attendant decreases in accumulation of these transcripts and their subsequent translation products.

Our studies confirm the finding of Murgia *et al*. (1993) that oATP treatment can reduce ecto-ATPase activity. Although those authors found that oATP modestly (~40%) inhibited the ecto-ATPase activity of murine macrophages, we observed a much more efficacious action (~90% inhibition) in the human 1321N1 astrocytes. The inhibition of extracellular nucleotide metabolism by oATP caused accumulation of extracellular nucleotides presumably released by constitutive or mechanically stimulated pathways, as described in several cell types including 1321N1 astrocytes (Beigi & Dubyak, 2000; Lazarowski *et al*., 2000; Joseph *et al*., 2003).

Repression of extracellular nucleotide scavenging and consequent elevation of extracellular nucleotide levels may affect both P2 receptor-dependent and P2 receptor-independent responses. For example, a constitutive desensitization of $P2Y_1$ receptors has been observed in KG-1 myeloblasts, which lack expression of native ecto-ATPases (Clifford *et al*., 1997) and in platelets isolated from CD39-knockout mice (Enjyoji *et al*., 1999). The attenuation of $P2Y_1R$ responses that we observed in oATP-treated HEK293 cells was in part due to a similar desensitization mechanism. Ecto-nucleotidases also play important roles in the scavenging of extracellular nucleotides for reuptake as nucleosides and resynthesis as intracellular nucleotides (Zimmermann, 2000). Disruption of this nucleotide-recycling loop by oATP for several hours may reduce the overall efficiency of fundamental intracellular processes such as nucleic acid synthesis. Depending on cellular background, this could be reflected in a reduced rate of DNA replication, transcription to RNA, or cell proliferation and/or survival. Craighead *et al*. (2001) recently described a potent toxic effect of oATP on the survival of rat cerebellar granule neurons placed in primary tissue cultures. Bradley & Bradley (2001) have shown that elevated levels of extracellular ATP and adenosine markedly inhibit proliferation of the P2 receptor-null 1321N1 astrocytes, and that these actions were reversed by nucleoside transport inhibitors. Our observation that oATP attenuates both ecto-ATPase activity and NF-κB-regulated gene expression in these 1321N1 astrocytes further supports a role for disruption of fundamental nucleotide recycling pathways in the pleiotropic actions of this reactive nucleotide analog on inflammatory cell function.

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