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Potentiation of cytokine induction of group IIA phospholipase A_2 in rat mesangial cells by ATP and adenosine *via* the A2A adenosine receptor

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1 In rat mesangial cells extracellular nucleotides were found to increase arachidonic acid release by a cytosolic phospholipase A_2 through the $P2Y_2$ purinergic receptor.

2 In this study we investigated the effects of ATP and UTP on interleukin-1β (IL-1β)-induced mRNA expression and activity of group IIA phospholipase A_2 ($sPLA_2$-IIA) in rat mesangial cells.

3 Treatment of cells for 24 h with extracellular ATP potentiated IL-1β-stimulated $sPLA_2$-IIA induction, whereas UTP had no effect.

4 We obtained the following evidence that the $P2Y_2$ receptor is not involved in the potentiation of $sPLA_2$-IIA induction: (i) ATP-γ-S had no enhancing effect; (ii) suramin, a P_2 receptor antagonist, did not inhibit ATP-mediated potentiation; (iii) inhibition of degradation of extracellular nucleotides by the 5′-ectonucleotidase inhibitor AOPCP did not enhance $sPLA_2$-IIA induction and (iv) adenosine deaminase treatment completely abolished the ATP-mediated potentiation of $sPLA_2$-IIA induction.

5 In contrast, treatment of mesangial cells with adenosine or the A2A receptor agonist CGS 21680 mimicked the effects of ATP in enhancing IL-1β-stimulated $sPLA_2$-IIA induction, whereas the specific A2A receptor antagonist ZM 241385 completely abolished the potentiating effect of ATP or adenosine.

6 The protein kinase A inhibitor Rp-8-Br-cyclic AMPS dose-dependently inhibited the enhancing effect of ATP or adenosine indicating the participation of an adenosine receptor-mediated cyclic AMP-dependent signalling pathway.

7 These data indicate that ATP mediates proinflammatory long-term effects in rat mesangial cells *via* its degradation product adenosine through the A2A receptor resulting in potentiation of $sPLA_2$-IIA induction.

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Abbreviations: BSA, bovine serum albumin; DMEM, Dulbecco's modified essential medium; IL-1β, interleukin-1β; NO, nitric oxide; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; $sPLA_2$-IIA, group IIA phospholipase A_2

Introduction

Extracellular nucleotides play an important role in the regulation of pathophysiological processes in the kidney. Nucleotides are released into the extracellular space upon injury of glomerular cells and thrombocyte aggregation and may reach high local concentrations (Gordon, 1986). Significant proinflammatory activities of extracellular adenine nucleotides were found during anti-Thy-1-glomerulonephritis, where the percentage of oxygen radical-producing granulocytes was markedly increased in glomeruli after treatment of nephritic rats with ATP-γ-S (Poelstra *et al*., 1992). Studies in glomerular mesangial cell cultures show multiple mitogenic reactions to ATP or UTP such as activation of phosphatidylinositol-bisphosphate-specific phospholipase C with subsequent increase in inositoltrisphosphate formation and intracellular Ca^{2+} (Pfeilschifter, 1990; Pavenstädt *et al*., 1993), activation of protein kinase C (PKC) and synthesis of prostaglandin E_2 (Pfeilschifter, 1990) activation of phospholipase D (Pfeilschifter & Merriweather, 1993), activation of the classical p42/p44 mitogen-activated protein (MAP) kinases (Huwiler & Pfeilschifter, 1994) as well as stress-activated protein kinases (Huwiler *et al*., 1997, 2000). It was suggested that ATP and UTP exert their equipotent actions through a common nucleotide receptor ($P2Y_2$; Pfeilschifter, 1990). Extracellular ATP and UTP stimulate the proliferation of mesangial cells *via* P2Y receptors (Huwiler & Pfeilschifter, 1994; Harada *et al*., 2000) raising the possibility that they act as growth factors and may promote mesangial cell proliferation during glomerular diseases (for review, see Schulze-Lohoff *et al*., 1996).

In contrast to these immediate effects of ATP and UTP little is known about long-term effects of these nucleotides on proinflammatory enzymes, which are induced several hours after cytokine-treatment of mesangial cells. In this respect

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Mohaupt *et al*. (1998) described that activation of purinergic $P2Y_2$ receptors by ATP or UTP inhibits 24-h nitrite production which derived from the LPS-stimulated induction of inducible nitric oxide synthase. The inhibition by ATP was shown to be mediated by a PKC-dependent mechanism, as already previously suggested by Mühl & Pfeilschifter (1994).

Another enzyme, which is induced at the transcriptional level in rat mesangial cells, is the group IIA phospholipase A_2 ($sPLA_2$-IIA; for review, see Pfeilschifter *et al*., 1997). Similar to inducible nitric oxide synthase, the expression of $sPLA_2$-IIA is stimulated by proinflammatory cytokines such as interleukin-1β (IL-1β) or by cyclic AMP-elevating agents such as forskolin and is secreted into the cell culture supernatant of these cells (Walker *et al*., 1995, 1998; Pfeilschifter *et al*., 1993, 1997). Both compounds act synergistically in inducing $sPLA_2$-IIA expression. Moreover, we recently described that endogenous NO is a potent mediator of $sPLA_2$-IIA gene transcription (Rupprecht *et al*., 1999). We also found that $sPLA_2$-IIA is downregulated by PKC-activating compounds such as phorbol 12-myristate 13-acetate (PMA) or thrombin as a physiological agonist (Scholz *et al*., 1999).

Since extracellular nucleotides also activate PKC (Pfeilschifter, 1990; Pfeilschifter & Huwiler, 1996), we expected that ATP or UTP would also inhibit the IL-1β-stimulated induction of $sPLA_2$-IIA. Surprisingly, we found that extracellular ATP as well as adenosine, but not UTP potentiated the cytokine-stimulated induction of $sPLA_2$-IIA. In this study we obtained evidences that extracellular ATP is rapidly degraded to adenosine by 5′-ectonucleotidase and then exerts long-term effects *via* the A2A adenosine receptor and subsequent activation of the cyclic AMP-mediated pathways.

Methods

Cell culture

Rat renal mesangial cells were cultured as described previously (Pfeilschifter *et al*., 1984). The cells were grown in RPMI 1640 supplemented with 10% foetal calf serum, penicillin (100 u ml^{-1}), streptomycin (100 μg ml^{-1}) and bovine insulin (0.66 u ml^{-1}). For experiments cells were cultured in plastic Petri dishes (Greiner, Frickenhausen, Germany) with 3.5 cm or 10 cm diameter to near confluency. Then cells were incubated for 24 h in serum-free DMEM containing 0.1 mg ml^{-1} fatty acid-free bovine serum albumin (BSA). After this period cells were treated for 24 h with the compounds to be tested.

$sPLA_2$ assay

$sPLA_2$ activity in the supernatant of mesangial cell cultures was determined with [1-^{14}C]-oleate-labelled *Escherichia coli* as substrate as described previously (Märki & Franson, 1986). Briefly, assay mixtures (1 ml) contained 100 mM Tris/HCl (pH 7.0), 1 mM $CaCl_2$, [1-^{14}C]-oleate-labelled *E. coli* ($\approx$5000 c.p.m.) and 5 μl of the enzyme-containing supernatants of the cell cultures, which is sufficient to produce less than 5% substrate hydrolysis to be in a linear range. Reaction mixtures were incubated for 1 h at 37°C in a thermomixer. The extraction of the lipids was performed by the Dole method exactly as described (Pfeilschifter *et al*., 1993). Free [1-^{14}C]-oleate was measured in a β-counter.

Northern blot analysis

From confluent mesangial cells cultured in 10 cm diameter culture dishes total cellular RNA was extracted from the cell pellets using the guanidinium isothiocyanate/phenol/chloroform method (Sambrook *et al*., 1989). Samples of 20 μg of RNA were separated on 1.4% agarose/formaldehyde gels and transferred to a gene screen membrane. After u.v. cross-linking and prehybridization for 4 h the filters were hybridized for 16 h at 42°C to [^{32}P]-labelled cDNA insert from $sPLA_2$-IIA. DNA probes were radioactively labelled with [α-^{32}P]-dCTP by random priming. Finally, the filters were washed twice with 2× sodium chloride (3 M)/sodium citrate (0.3 M; SSC)/0.1% sodium dodecyl sulphate (SDS) for 2×20 min and several times at 65°C with 0.2×SSC/1% SDS. The signal was detected and quantified with a phosphorimager BAS 1500 from Fuji (Raytest, Straubenhardt, Germany). To correct for variations in RNA loading the respective cDNA probes were stripped and the blots were rehybridized to the [α-^{32}P]-dCTP-labelled cDNA insert for GAPDH. The amount of mRNA calculated for $sPLA_2$-IIA in IL-1β-stimulated cells is expressed as 100%. The numbers at the top of the Northern blot represent the corrected density expressed as the percentage of RNA found in IL-1β stimulated cells.

Data presentation

The mean of the values of $sPLA_2$-activity measured after IL-1β treatment of mesangial cells was calculated as 100%. The data represent the means of several independent determinations ± s.e.mean ($n=3$) as indicated in the figure legends.

Statistical analysis was performed by Student's *t*-test to determine significant differences among two groups, or by repeated-measures ANOVA followed by Dunnett's test comparing all concentrations of the respective compounds to be tested with IL-1β plus ATP or plus adenosine. A $P<0.05$ was defined as significant.

Materials

Recombinant human interleukin-1β was obtained from Cell Concepts (Umkirch, Germany). [1-^{14}C]-oleic acid, and [α-^{32}P]-dCTP were from Amersham-Pharmacia, Freiburg, Germany. ATP, UTP, adenosine, adenosine deaminase (EC 3.5.4.4) and Rp-8-Br-cAMPS (Rp-8-bromo-adenosine 3′, 5′-cyclic monophosphorothioate) were from Calbiochem. ZM 241385 (4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol) was provided by Tocris/Biotrend (Köln, Germany), and suramin, AOPCP (α,β-methylene adenosine 5′-diphosphate) and CGS 21680 (2-*p*-(2-carbocyethyl)phenethylamino-5′-N-ethylcarboxyamidoadenosine hydrochloride) by RBI/Sigma (München, Germany). Nylon membranes (Gene Sreen) were purchased from NEN Life Science (Köln, Germany). All cell culture media and nutrients were from Gibco BRL (Eggenstein, Germany), and all other

chemicals used were from either Merck (Darmstadt, Germany), Sigma (Munich, Germany) or Fluka (Deisenhofen, Germany).

Results

Effect of extracellular ATP and UTP on IL-1β-stimulated sPLA$_2$-IIA mRNA induction, secretion and activity

Recently we have shown that the PKC-activating compounds thrombin or phorbol ester PMA inhibit IL-1β-stimulated sPLA$_2$-IIA induction (Scholz *et al*., 1999). ATP and UTP are potent mitogens and activators of PKC in rat mesangial cells (Pfeilschifter & Huwiler, 1996). The scope of this study was to investigate effects of extracellular nucleotides on sPLA$_2$-IIA expression.

In rat mesangial cells sPLA$_2$-IIA is induced at the transcriptional level about 8 h after IL-1β treatment reaching maximal mRNA and protein levels after 24 h (Pfeilschifter *et al*., 1989; Schalkwijk *et al*., 1991). Therefore, cells were treated for 24 h with IL-1β in absence or presence of ATP or UTP, each 100 μM, which usually elicits maximal responses in the diverse signal transduction pathways in mesangial cells (Huwiler *et al*., 2000).

Surprisingly, Northern blot analysis showed that the IL-1β-stimulated induction of sPLA$_2$-IIA mRNA expression was markedly enhanced by ATP, whereas UTP had no potentiating effects (Figure 1A). This Northern blot is representative for five different experiments with similar results. On average the increase in IL-1β-stimulated sPLA$_2$-IIA mRNA by 100 μM ATP was 2 fold$\pm$0.4 ($n=5$; $P<0.05$). ADP and AMP (each 100 μM) had comparable effects (data not shown).

ATP, when given alone, had a stimulatory effect on the mRNA expression of sPLA$_2$-IIA, which was 30%$\pm$15 ($n=5$; $P<0.05$) of the level reached by IL-1β.

From the cell culture supernatants of these experiments sPLA$_2$ activity assays were performed. Since sPLA$_2$-IIA is the major enzyme secreted by rat mesangial cells (Schalkwjik *et al*., 1992; Scholz-Pedretti *et al*., 2000; van der Helm *et al*., 2000), sPLA$_2$ activity measured in this cell system can be used as a read-out for secreted sPLA$_2$-IIA in the following experiments. In five different experiments it was found that sPLA$_2$-IIA activity was induced after ATP treatment alone by 50$\pm$4% of the level reached by IL-1β ($n=3$; $P<0.05$). A combination with IL-1β and ATP potentiated sPLA$_2$-IIA activity 2 fold$\pm$0.65 ($n=3$; $P<0.01$), when compared to IL-1β alone. In contrast, UTP did not change the IL-1β-stimulated effects and had no effects when given alone (Figure 1B). These data correspond to the sPLA$_2$-IIA mRNA expression pattern (Figure 1A).

Effect of ATP-γ-S on IL-1β-stimulated sPLA$_2$-IIA induction

To test whether a non-hydrolysable analogue of ATP has a similar potentiating effect on sPLA$_2$-IIA mRNA induction and activity, we coincubated mesangial cells for 24 h with ATP-γ-S (100 μM). In contrast to ATP the stable compound ATP-γ-S alone had no effect on sPLA$_2$-IIA induction and did not enhance IL-1β-stimulated sPLA$_2$-IIA mRNA expression (Figure 2A) and activity (Figure 2B).

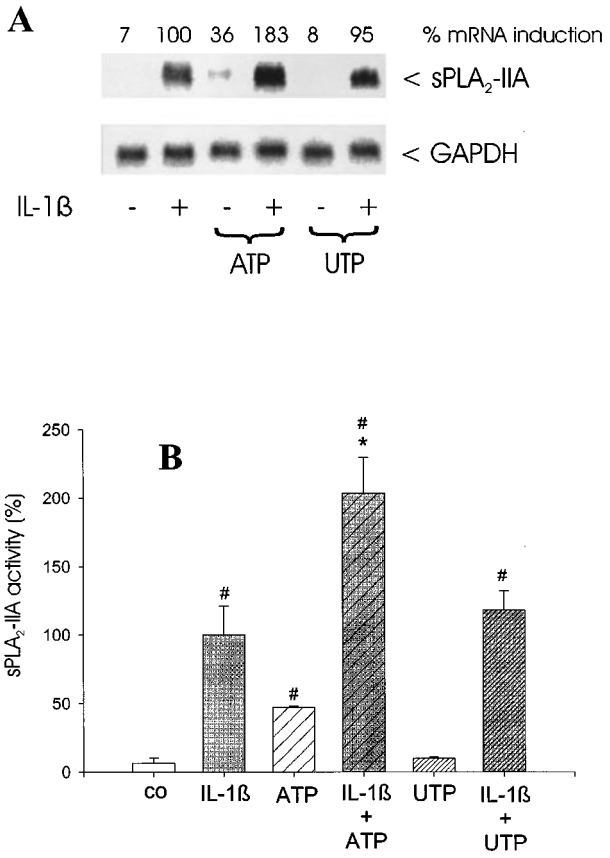

Figure 1 Effect of ATP and UTP on IL-1β-stimulated sPLA$_2$-IIA induction. Mesangial cells were treated for 24 h with ATP or UTP (each 100 μM) in the absence or presence of IL-1β (1 nM) as indicated. (A) Northern blots were performed from the RNA extracts of the cells as described in Methods. To correct for differences in loading, the signal density of each RNA sample hybridized was divided by that hybridized to the GAPDH probe. The amount of mRNA calculated for sPLA$_2$-IIA in IL-1β-stimulated cells is expressed as 100%. The numbers at the top of the Northern blot represent the corrected density expressed as the percentage of RNA found in IL-1β stimulated cells. This is a representative Northern blot out of five separate experiments with comparable results. (B) sPLA$_2$-IIA activity was determined as described in Methods. The means of the values were calculated from five different experiments ($n=3$). The mean value obtained for IL-1β is indicated as 100%$\pm$s.e.mean. Significant differences from control (without stimulation): #$P<0.05$; ANOVA. Significant differences from IL-1β stimulation (without nucleotide): *$P<0.05$; ANOVA.

Effect of the P2 receptor antagonist suramin

Suramin, a putative P$_2$ receptor antagonist (Hoiting *et al*., 1990), was found to almost completely block ATP and UTP-induced activation of the p42/44-MAP kinase (Huwiler & Pfeilschifter, 1994) as well as the p38-MAP kinase cascade (Huwiler *et al*., 2000). To investigate whether the ATP-induced enhancement of IL-1β-stimulated sPLA$_2$-IIA induction is mediated *via* P$_2$ receptors, mesangial cells were treated for 24 h with ATP and IL-1β in presence of suramin (100 μM).

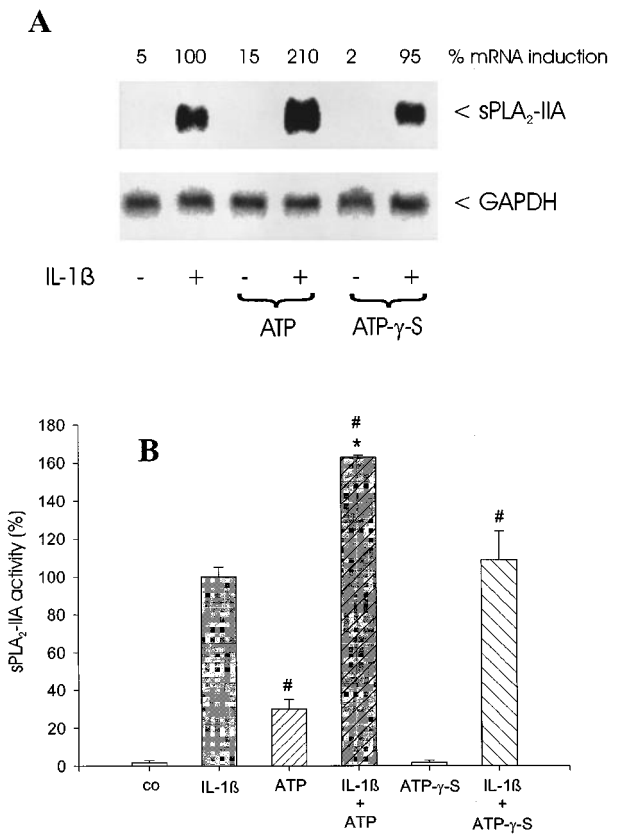

Figure 2 Effect of ATP-γ-S on IL-1β-stimulated sPLA$_2$-IIA induction. Mesangial cells were treated for 24 h with IL-1β (1 nM) in the absence or presence of ATP (100 μM) or ATP-γ-S (100 μM) as indicated. (A) Northern blot analysis was performed three times with comparable results and quantification was performed as described in Figure 1. This is a representative Northern blot out of three different experiments with comparable results. (B) sPLA$_2$-IIA activity assay was performed as described in Methods. The means of the values were calculated from three different experiments ($n=3$). The mean value obtained for IL-1β is indicated as 100% $\pm$ s.e.mean. Significant differences from control (without stimulation): #$P<0.05$; Student's t-test. Significant differences from IL-1β stimulation (without nucleotide): *$P<0.05$; Student's t-test.

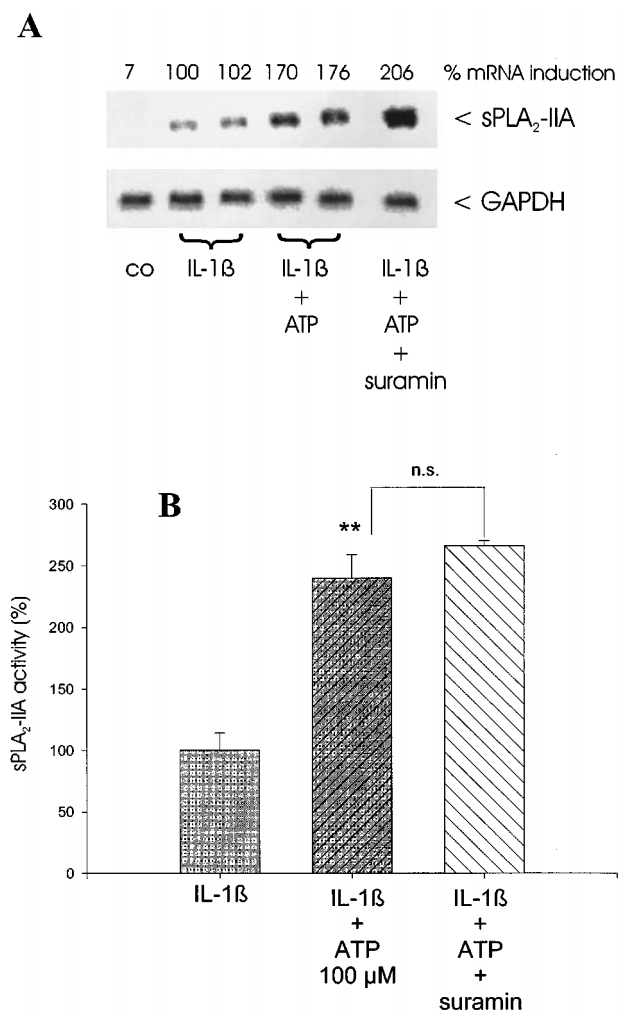

Figure 3 Effect of suramin on ATP potentiation of IL-1β-stimulated sPLA$_2$-IIA expression. Mesangial cells were incubated for 24 h with IL-1β (1 nM) in the absence or presence of ATP (100 μM) or ATP plus suramin (each 100 μM) as indicated. (A) Northern blot analysis was performed three times with comparable results and quantification was performed as described in Figure 1. This is a representative Northern blot out of three different experiments with comparable results. (B) sPLA$_2$-IIA activity assay was performed as described in Methods. The means of the values were calculated from three different experiments ($n=3$). The mean value obtained for IL-1β is indicated as 100% $\pm$ s.e.mean. Significant differences from IL-1β stimulation (without nucleotide): **$P<0.01$; Student's t-test; n.s.–not significant.

The data in Figure 3 show that suramin had no inhibitory effect on the ATP-mediated enhancement of IL-1β-stimulated sPLA$_2$-IIA mRNA expression (Figure 3A) and activity (Figure 3B). In some experiments even an enhancement of sPLA$_2$-IIA mRNA induction was observed, which might be due to unspecific actions of suramin (for review, see Ralevic & Burnstock, 1998). Suramin had no effects on sPLA$_2$-IIA expression in unstimulated or IL-1β-treated mesangial cells (data not shown).

Effect of inhibition of 5′-ectonucleotidase

Mesangial cells express a 5′-ectonucleotidase activity at the cell surface, which rapidly degrades exogenous AMP to adenosine (Le Hir & Kaissling, 1993; Stefanovic *et al*., 1993). To prevent the formation of adenosine from exogenously added ATP we incubated mesangial cells with AOPCP (100 μM), an inhibitor of 5′-ectonucleotidase activity, thereby inhibiting AMP metabolism to adenosine.

The results show that the treatment of the cells with AOPCP completely abolished the ATP-mediated enhancement of IL-1β-stimulated sPLA$_2$-IIA mRNA expression (Figure 4A) and activity (Figure 4B). AOPCP had no effect on sPLA$_2$-IIA expression of unstimulated or IL-1β-treated mesangial cells (data not shown).

We therefore concluded that the potentiating effect of ATP on sPLA$_2$-IIA induction is not due to a direct ATP action, but is caused by its degradation product adenosine, which exerts its effects *via* specific adenosine receptors and signalling pathways distinct from ATP (for review, see Ralevic & Burnstock, 1998).

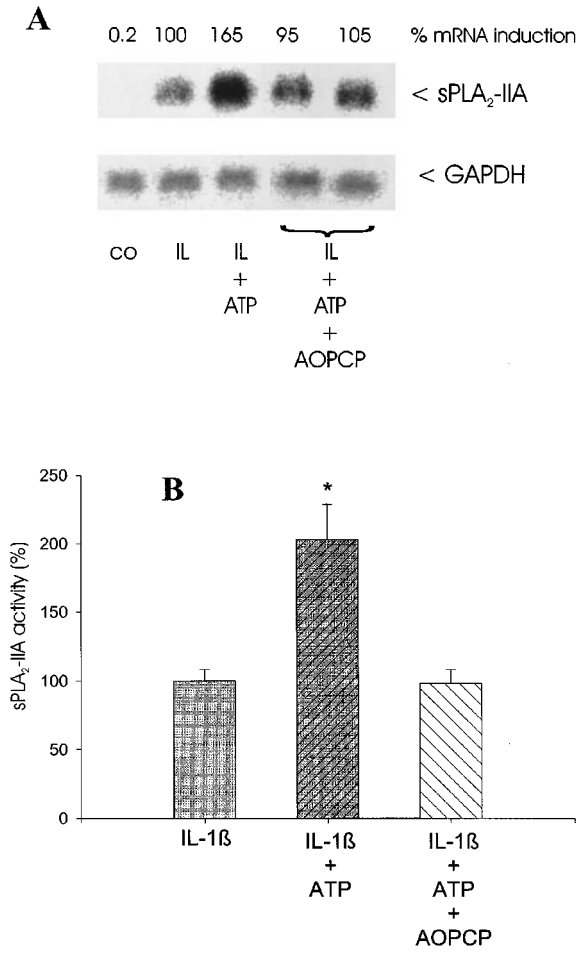

Figure 4 Effect of AOPCP on ATP potentiation of IL-1β-stimulated $sPLA_2$-IIA expression. Mesangial cells were incubated for 24 h with IL-1β (1 nM) in the absence or presence of ATP (100 μM) or ATP plus AOPCP (100 μM) as indicated. (A) Northern blot analysis and quantification of mRNA expression was performed as described in legend of Figure 1. This is a representative Northern blot out of three different experiments with comparable results. (B) $sPLA_2$-IIA activity was determined in supernatants of mesangial cell cultures. The means of the values were calculated from three different experiments ($n=3$). The mean value obtained for IL-1β is indicated as 100% $\pm$ s.e.mean. Significant differences from IL-1β stimulation (without nucleotide): $^*P<0.05$; Student's t-test.

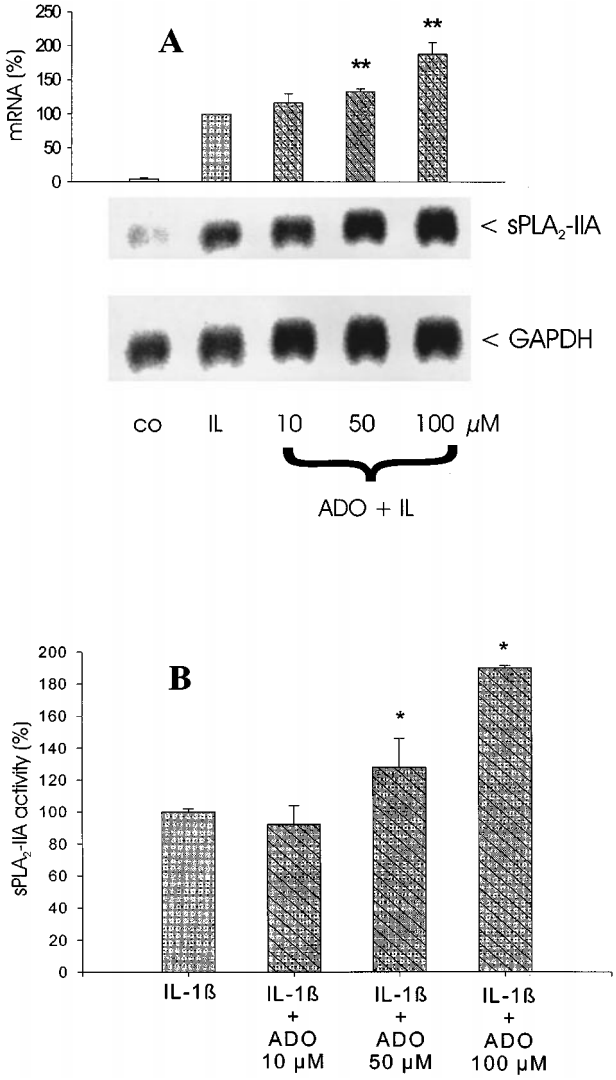

Figure 5 Effect of adenosine on IL-1β-stimulated $sPLA_2$-IIA induction. Mesangial cells were incubated for 24 h with IL-1β (1 nM) in the absence or presence of the indicated adenosine (ADO) concentrations. (A) Northern blot analysis and quantification of mRNA expression was performed as described in legend of Figure 1. This is a representative Northern blot out of four different experiments with comparable results. The bars represent the mean values of mRNA induction (%) from four independent experiments $\pm$ s.e.mean. The mean value obtained for IL-1β is indicated as 100% $\pm$ s.e.mean. Significant differences from IL-1β stimulation (without adenosine): $^{**}P<0.01$; ANOVA. (B) $sPLA_2$-IIA activity was determined in supernatants of mesangial cell cultures. The means of the values were calculated from three different experiments ($n=3$). The mean value obtained for IL-1β is indicated as 100% $\pm$ s.e.mean. Significant differences from IL-1β stimulation (without adenosine): $^*P<0.05$; ANOVA.

Effect of adenosine on IL-1β-stimulated sPLA2-IIA induction

To test if adenosine has a similar potentiating effect as ATP on IL-1β-stimulated $sPLA_2$-IIA induction, mesangial cells were treated with different concentrations of adenosine in the presence of IL-1β for 24 h.

Analysis of mRNA induction and activity shows that adenosine concentration-dependently potentiated the IL-1β-stimulated mRNA expression (Figure 5A). In five independent experiments the average increase in $sPLA_2$-IIA mRNA induction was 1.8 fold $\pm$ 0.4; $P<0.05$). In the same experiments an average increase in $sPLA_2$-IIA activity (Figure 5B) was observed by 1.9 fold $\pm$ 0.35 ($n=3$; $P<0.01$). This indicates that adenosine mimicked ATP in enhancing IL-1β-stimulated $sPLA_2$-IIA induction in mesangial cells. Adenosine, when given alone, had a stimulatory effect on $sPLA_2$-IIA by 2.5 fold $\pm$ 1 of control values ($P<0.05$; $n=3$; data not shown).

Effect of adenosine deaminase

To confirm that ATP mediates its effects on IL-1β-stimulated $sPLA_2$-IIA induction *via* adenosine, we incubated mesangial cells with IL-1β and ATP in the presence of adenosine deaminase. This enzyme degrades adenosine,

which may have arisen from metabolism of ATP. As control we treated the cells with adenosine deaminase in the presence of adenosine. The data in Figure 6 show that adenosine deaminase dose-dependently abolished the potentiating effect of ATP as well as adenosine on IL-1β-induced $sPLA_2$-IIA activity. This observation strongly supports our hypothesis that ATP mediates its effect on $sPLA_2$-IIA induction through its degradation product adenosine. Adenosine deaminase had no effect on $sPLA_2$-IIA expression in unstimulated or IL-1β-treated mesangial cells (data not shown).

Effect of inhibition of cyclic AMP-mediated signal transduction

Binding of adenosine to A_2 receptors of mesangial cells results in activation of adenylate cyclase and subsequent increase in cyclic AMP (Olivera & Lopez-Novoa, 1992). Earlier studies in rat mesangial cells have shown (i) that cyclic AMP induces $sPLA_2$-IIA mRNA induction and (ii) that cyclic AMP acts synergistically with IL-1β in this respect (Walker *et al*., 1995; Pfeilschifter *et al*., 1997).

To further support our hypothesis that ATP acts *via* an A_2 receptor-mediated increase in cyclic AMP and subsequent signal transduction *via* protein kinase A we treated mesangial cells for 24 h with Rp-8-Br-cAMPS, a potent inhibitor of protein kinase A (Gjertsen *et al*., 1995).

The data in Figure 7 show that Rp-8-Br-cAMPS dose-dependently reduced the synergistic interaction of IL-1β and ATP or adenosine on $sPLA_2$-IIA mRNA expression (Figure 7A) and activity (Figure 7B), reaching the level of IL-1β at 30 μM of the inhibitor. These results demonstrate that ATP and adenosine mediate their potentiating effect on IL-1β-stimulated $sPLA_2$-IIA induction *via* cyclic AMP-activated signal transduction pathways. Rp-8-Br-cAMPS had no effect on $sPLA_2$-IIA expression in unstimulated cells and it did not inhibit IL-1β-stimulated $sPLA_2$-IIA induction (data not shown), thus confirming earlier observations that IL-1β-mediated $sPLA_2$-IIA expression is independent of cyclic AMP-mediated signal transduction (Pfeilschifter *et al*., 1991).

Effect of CGS 21680 as a specific A2A receptor agonist

We next tested CGS21680, which is a 140 fold selective agonist for the A2A receptor subtype versus the A_1 receptor (Hutchison *et al*., 1990) and which binds only with a low affinity to the A2B receptor.

The data in Figure 8 show that CGS21680 dose-dependently enhanced the effect on IL-1β-stimulated $sPLA_2$-IIA activity thus indicating that in mesangial cells the activation of the A2A receptor subtype may represent the mechanism by which ATP or adenosine mediate their

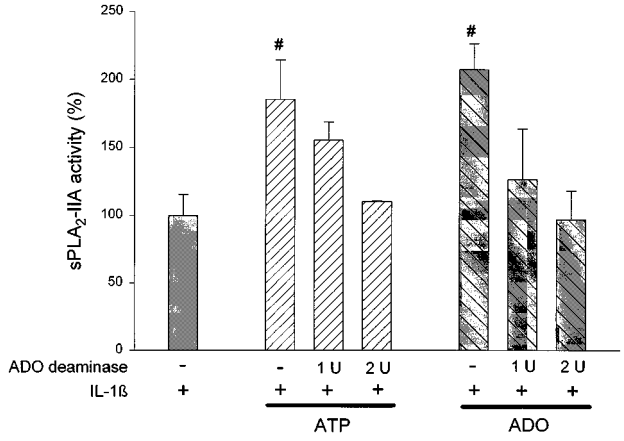

Figure 6 Effect of adenosine deaminase on ATP- or adenosine-mediated potentiation of IL-1β-stimulated $sPLA_2$-IIA expression. Mesangial cells were incubated for 24 h with IL-1β (1 nM) in the absence or presence of ATP or adenosine (ADO), (each 100 μM) plus adenosine deaminase (1 or 2 u/ml^{-1}). $sPLA_2$-IIA activity was determined in the cell culture supernatants as described in Methods. The values represent the means of three independent experiments $\pm$ s.e.mean. ($n = 3$). The mean value obtained for IL-1β is indicated as 100% $\pm$ s.e.mean. Significant differences from IL-1β stimulation alone: $^*P < 0.05$; ANOVA.

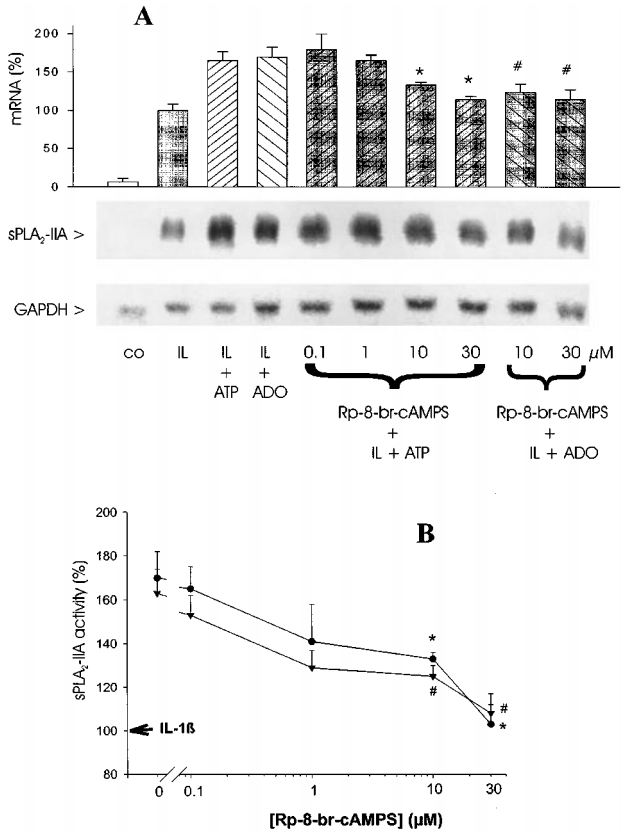

Figure 7 Effect of the protein kinase A inhibitor Rp-8-Br-cAMPS on ATP- or adenosine-mediated potentiation of IL-1β-stimulated $sPLA_2$-IIA expression. Mesangial cells were treated for 24 h with IL-1β (1 nM) in the absence or presence of ATP or adenosine (ADO) and the indicated concentrations of Rp-8-Br-cAMPS. (A) Northern blot analysis and quantification of mRNA expression was performed as described in legend of Figure 1. This is a representative Northern blot out of four different experiments with comparable results. The bars represent the mean values of mRNA induction (%) from four independent experiments $\pm$ s.e.mean. The mean value obtained for IL-1β is indicated as 100% $\pm$ s.e.mean. (B) $sPLA_2$-IIA activity was determined in the cell culture supernatants as described in Methods. The values represent the means of three independent experiments $\pm$ s.e.mean. ($n = 3$). The mean value obtained for IL-1β is indicated as 100% $\pm$ s.e.mean. Significant differences from corresponding IL-1β plus ATP stimulation (without inhibitor): $^*P < 0.001$; ANOVA. Significant differences from corresponding IL-1β plus adenosine stimulation (without inhibitor): $\#P < 0.001$; ANOVA.

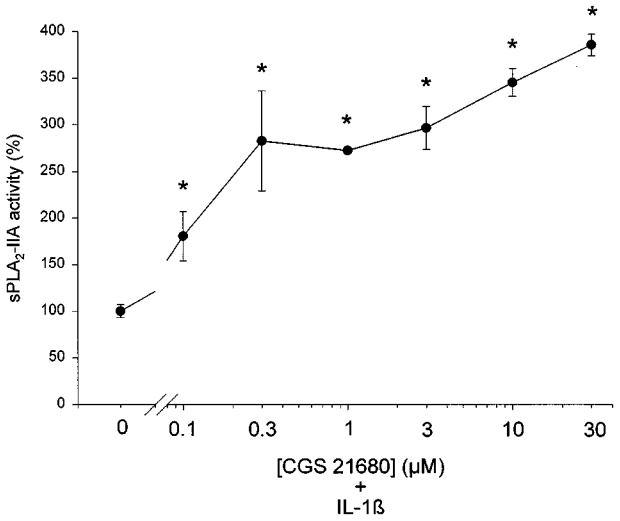

Figure 8 Effect of the specific A2A adenosine receptor agonist CGS 21680 on IL-1β-stimulated $sPLA_2$ activity. Mesangial cells were treated for 24 h with IL-1β (1 nM) in the absence or presence of the indicated concentrations of CGS 21680. $sPLA_2$-IIA activity in the cell culture supernatants was detected as described in Methods. The means of the values were calculated from two different experiments ($n=3$). The mean value obtained for IL-1β is indicated as 100% ± s.e.mean. Significant differences from IL-1β stimulation alone: $^*P<0.05$; ANOVA.

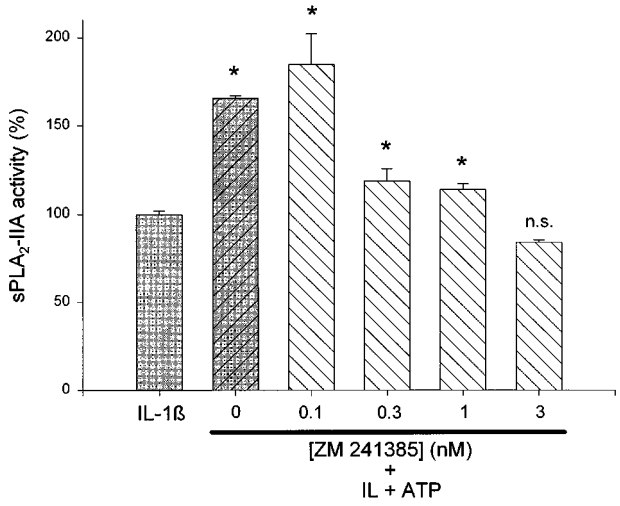

Figure 9 Effect of the specific A2A adenosine receptor antagonist ZM 241385 on the ATP potentiation of IL-1β-stimulated $sPLA_2$-IIA expression. Mesangial cells were incubated for 24 h with IL-1β (1 nM) in the absence or presence of ATP plus ZM 241385 at the indicated concentrations. $sPLA_2$-IIA activity in the cell culture supernatants was detected as described in Methods. The means of the values were calculated from two different experiments ($n=3$). The mean value obtained for IL-1β is indicated as 100% ± s.e.mean. Significant differences from IL-1β stimulation alone: $^*P<0.05$; ANOVA, n.s.– not significant.

potentiating effects on IL-1β-stimulated $sPLA_2$-IIA induction. CGS21680 alone did not induce $sPLA_2$-IIA mRNA induction and activity (data not shown).

Effect of ZM 241385 as a specific A2A adenosine receptor antagonist

Further support for the involvement of the A2A receptor in the effect of ATP on $sPLA_2$-IIA induction was obtained with ZM 241385, a non-xanthine, selective A2A receptor antagonist (Poucher *et al*., 1995; Keddie *et al*., 1996), which dose-dependently reduced the ATP-mediated potentiation of IL-1β-stimulated $sPLA_2$-IIA activity reaching the level of IL-1β at 1–3 nM (Figure 9). ZM 241385 had no effect on $sPLA_2$-IIA induction in unstimulated or IL-1β-treated mesangial cells (data not shown).

Discussion

In this study we have shown for the first time that long-term treatment of rat mesangial cells with extracellular ATP potentiated the IL-1β induction of $sPLA_2$-IIA. At a first glance this result was surprising, since we expected that ATP, which is known to activate PKC in mesangial cells (Pfeilschifter & Huwiler, 1996), would inhibit the induction of $sPLA_2$-IIA. We have shown earlier that activators of PKC drastically reduce IL-1β-stimulated mRNA expression of $sPLA_2$-IIA in mesangial cells (Scholz *et al*., 1999). In this respect, it was also surprising, that UTP, which shows an equipotent potency to ATP in activating PKC *via* $P2Y_2$ purinergic receptors (Pfeilschifter, 1990; Pfeilschifter & Huwiler, 1996) had no effect on $sPLA_2$-IIA induction, when coincubated for 24 h with IL-1β. Recently, additional P_2 receptor subtypes were described in mesangial cells (Harada *et al*., 2000). P2Y4, which was shown to be equally sensitive to UTP and ATP (Bogdanov *et al*., 1998), or $P2Y_6$ (Huwiler *et al*., 2000), which is activated most potently by UDP, but not by UTP (Nicholas *et al*., 1996). However, a rapid desensitization of purinergic receptors (Pfeilschifter, 1990), which might be the reason for the lack of an effect of UTP on $sPLA_2$-IIA induction, or consequences of long-term effects of UTP on signal transduction mechanisms in mesangial cells were not investigated so far.

From the fact that ATP potentiated rather than inhibited $sPLA_2$-IIA induction by IL-1β, we inferred that the long-term effects of ATP observed in this study are not primarily mediated *via* P_2 receptor-activated signalling pathways. This was corroborated by the following observations: (i) the non-hydrolysable ATP-analogue ATP-γ-S, which binds to different P_2 receptor subtypes, had no potentiating effects on $sPLA_2$-IIA induction; (ii) the P2Y antagonist suramin did not abolish the ATP-mediated potentiation; (iii) the inhibition of 5′-ectonucleotidase activity with AOPCP, which may lead to an accumulation of extracellular AMP, did not potentiate IL-1β-stimulated $sPLA_2$-IIA induction and (iv) treatment of cells with ATP and IL-1β in the presence of adenosine deaminase completely abolished the ATP-mediated potentiation.

Mesangial cells express 5′-ectonucleotidase activity at the cell surface, which represents the major source of extracellular adenosine in the kidney (Stefanovic *et al*., 1993; Le Hir & Kaissling, 1993). This ATP metabolite acts *via* adenosine-specific receptors and signalling cascades different from P_2 receptors (Ralevic & Burnstock, 1998). In mesangial cells the presence of A_1 and A_2 adenosine receptors was characterized by subtype-specific agonists (Olivera & Lopez-Novoa, 1992; Stefanovic & Valhovic, 1995). The activation of A_1 adenosine receptor results in inhibition of adenylate

cyclase causing a decrease in cellular cyclic AMP levels, whereas A_2 receptors couple *via* G_S to adenylate cyclase and trigger an increase in cyclic AMP. In earlier studies cyclic AMP was shown to stimulate sPLA$_2$-IIA induction in rat mesangial cells and to act synergistically with IL-1β (Pfeilschifter *et al*., 1991) by using different signalling pathways and transcription factors, which control the induction of the enzyme by cyclic AMP and IL-1β (Walker *et al*., 1995, 1998; Pfeilschifter *et al*., 1997; Scholz-Pedretti *et al*., 2000). Refering to these observations we assumed that most probably the A_2 class of adenosine receptors rather than the A_1 adenosine receptor is involved in the observed effects on sPLA$_2$-IIA upregulation. Indeed we found a potentiating effect of CGS21680 which is an agonist specific for the A2A adenosine receptor on IL-1β-stimulated sPLA$_2$-IIA induction in mesangial cells, thus strongly suggesting that adenosine acts through the A2A adenosine receptor subtype and subsequent cyclic AMP-activated signalling to alter sPLA$_2$-IIA expression. This involves the activation of the cyclic AMP-dependent protein kinase A, which is responsible for phosphorylation and activation of transcription factors critical for the transcriptional regulation of genes in response to cyclic AMP (for review, see Daniel *et al*., 1998). Rp-8-Br-cAMPS, a potent inhibitor of protein kinase A, blocked the enhancing effects of ATP and adenosine on sPLA$_2$-IIA induction, thus again supporting the involvement of the A_2, but not the A_1 adenosine receptor in this process.

Further confirmation was obtained in experiments using the specific A2A receptor antagonists ZM 241385, which completely inhibited the ATP potentiation at a concentration of 3 nM (Figure 9).

Interestingly, using the xanthines DMPX (3,7-dimethyl-I-propargylxanthine) or CSC (8-(3-chlorostyryl)caffeine) as putative A_2 receptor antagonists we observed an enhancement rather than an inhibition of sPLA$_2$-IIA induction in a concentration range between 10 and 100 μM of these compounds (data not shown). Despite their increased selectivity as A_2 receptor antagonists they still may have the potential to inhibit phosphodiesterases, since they belong to the class of xanthates. This inhibition will result in an accumulation of cyclic AMP in amounts sufficient to potentiate the IL-1β-mediated sPLA$_2$-IIA induction. There are very few reports about effects of these xanthate derivatives distinct from their antagonistic effects on A_2 adenosine receptors. In this respect Pereira and coworkers (1998) showed that DMPX potentiated the isoproterenol-induced cyclic AMP increase without further discussing a possible mode of action of DMPX in this respect. Such an increase in cyclic AMP levels might be due to an inhibition of phosphodiesterases and thus would explain the synergistic effect of this compound or of CSC on IL-1β-stimulated sPLA$_2$-IIA induction in our system. In contrast, ZM 241385, which is a non-xanthate compound, does not inhibit phosphodiesterases (Poucher *et al*., 1995; Keddie *et al*., 1996) and therefore acts exclusively by blocking A2A receptors.

Adenosine as well as A2A agonists were described as potent inhibitors of inflammation in human embryonic kidney cells and in a rat model of ischemia-reperfusion injury (Okusa *et al*., 1999), as well as in anti-Thy 1 glomerulonephritis in the rat (Poelstra *et al*., 1992). In addition, adenosine was found to stimulate 5′-ectonucleotidase activity in rat mesangial cells *via* A_2 receptors (Stefanovic *et al*., 1993) thereby acting as a regulator of ATP levels by degrading elevated amounts of ATP during inflammatory processes in the kidney. Here we describe for the first time, that adenosine is a potent stimulator of the induction of sPLA$_2$-IIA which is a potent proinflammatory enzyme secreted by mesangial cells. This might be crucial for the mitogenic and proinflammatory effects of ATP, which itself is rapidly degraded, but which may act through its metabolite adenosine in long-term potentiation of pathophysiological processes in the glomerulus. Adenosine was described to stimulate proliferation in mesangial cells (MacLaughlin *et al*., 1997), thus participating in the progression of mitogenic signals induced by extracellular ATP. Moreover, A2A receptor-mediated cyclic AMP-signalling cascades may be involved in induction or potentiation of several other proinflammatory factors in mesangial cells, which are activated by cyclic AMP, such as the inducible nitric oxide synthase (Mühl *et al*., 1994; Eberhardt *et al*., 1998), cyclooxygenase 2 (Nüsing *et al*., 1996; Klein *et al*., 1998), or interleukin-6 (Dendorfer, 1996). Furthermore, cyclic AMP triggers apoptosis in mesangial cells (Mühl *et al*., 1996), which may be a critical step in the initiation as well as progression of glomerulonephritis.

Studies in patients with glomerulonephritis have shown a significant decrease of activity of adenosine deaminase in lymphocytes when compared to cells obtained from healthy individuals (Klinger *et al*., 1983). This may lead to an accumulation of adenosine and subsequent potentiation of proinflammatory cyclic AMP-mediated signal transduction pathways. A recent report shows that adenosine potentiates nitric oxide production *via* activation of inducible nitric oxide synthase in LPS-stimulated macrophages (Min *et al*., 2000). Moreover, the inhibition of adenosine degradation to inosine may prevent the activation of a negative feed-back mechanism, as inosine was found to inhibit cytokine formation in LPS-stimulated macrophages and thus may represent a potent endogenous antiinflammatory modulator (Hasko *et al*., 2000).

In summary, ATP seems to exert proinflammatory functions in rat mesangial cells in multiple ways: (i) as a short-lived agonist for P_2 receptors activating mitogen- and stress-mediated signalling cascades, and (ii) on a more long-term scale by its degradation to adenosine and subsequent potentiation of sPLA$_2$-IIA expression via A2A receptor-mediated signal transduction.

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