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P2Y-Receptors stimulating the proliferation of human mesangial cells through the MAPK$^{42/44}$ pathway

[1]Oliver Vonend, [1]Tobias Grote, [1]Vitus Oberhauser, [2]Ivar von Kügelgen & *,[1]Lars Christian Rump

[1]Department of Internal Medicine, Marienhospital Herne, Ruhr-University Bochum, Germany and [2]Department of Pharmacology, University Bonn, Germany

1 Mesangial cell proliferation is observed in a number of kidney diseases. The sympathetic cotransmitter ATP is suspected to play a major role in proliferative processes. Therefore, the effects of exogenous ATP on human mesangial cells in culture were studied.

2 Fresh human kidney cortex was processed to obtain mesangial cells in culture. Effects of nucleotides on [^{3}H]thymidine incorporation, the activation of mitogen-activated protein kinase and the cell number were studied. The involved P2-receptors were characterized pharmacologically. In addition, we searched for mRNA for P2Y- and P2X-receptors by RT–PCR.

3 ATP (0.1–300 μM) and related nucleotides induced a significant increase in [^{3}H]thymidine incorporation up to 220% of control. The adenine nucleotides ATP and ADP were about equally effective. Also ATP-γ-S, UTP, ADP-β-S and 2-m-thio-ADP induced a weaker response. UDP and α-β-methylene-ATP failed to induce an effect on [^{3}H]thymidine uptake.

4 ATP (100μM) induced a fast activation of the MAPK$^{42/44}$ pathway. The effects of ATP on MAPK$^{42/44}$ activation and [^{3}H]thymidine incorporation were reduced by the MAPK inhibitor PD 98059. Platelet-derived growth factor (PDGF 5 ng ml^{-1}) increased the cell number to more than 122% of control. ATP (10 μM) on top of PDGF amplified PDGF induced cell proliferation to 136% of control.

5 RT–PCR products for P2Y$_{1,2,4,6,11,12}$- and P2X$_{1,2,4,5,6,7}$-receptor subtypes were detected in human mesangial cells.

6 ATP has mitogenic effects on human mesangial cells. DNA synthesis is increased by the activation of the MAPK$^{42/44}$ pathway. ATP amplifies PDGF-induced cell hyperplasia.
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Keywords: P2Y-Receptors; ATP; UTP; sympathetic nervous system; human mesangial cells; proliferation; DNA synthesis; MAPK p42/44 activation

Abbreviations: ANOVA, analysis of variance; BSA, bovine serum albumin; ERK, extracellular signal-regulated kinase; FCS, foetal calf serum; HEPES, 4-(2-ydroxyethyl)-1-piperazineethanesulphonic acid; JNK, Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; mRNA, messenger ribonucleic acid; PBS, phosphate buffer saline; PDGF, plateled derived growth factor; RT, reverse transcription; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis

Introduction

Mesangial cells form the matrix of the glomerulus, hold the capillary arteries and connect them with the juxtaglomerular apparatus. They can influence the glomerular filtration rate by contracting the mesangium (Haas *et al*., 1999) and have been shown to release various growth hormones and cytokines; (Lee, 1995; Haas *et al*., 1999). In healthy individuals glomerular cells have a cell turnover of less than 1% (Pabst & Sterzel, 1983). However, in diabetic nephropathy, Lupusnephritis, IgA nephropathy and other renal diseases marked mesangial cell proliferation and increased extracellular matrix expansion has been observed. Since most of these patients develop end-stage renal failure within years, it is essential to understand the mechanisms involved in mesangial cell proliferation.

It is now well established that inhibition of the renin–angiotensin system and the sympathetic nervous system slows the decline of renal function in chronic renal failure (Rump *et al*., 2000; Hilgers & Mann, 2002). This is probably due to a reduction in local concentrations of growth promoting hormones and neurotransmitters, such as angiotensin II (Mezzano *et al*., 2001) and noradrenaline (Aizawa *et al*., 2001).

Accordingly, the sympatholytic drug moxonidine reduces renal damage independent from blood pressure effects in an experimental model of chronic renal failure (Amann *et al*., 2000). Since ATP is a cotransmitter of noradrenaline (Starke *et al*., 1991; Burnstock, 1995) in the kidney (Rump *et al*., 1996; Vonend *et al*., 2002), it is our hypothesis that P2-receptor activation by ATP contributes to progression of renal disease. In addition to the neuronal source of ATP, large amounts of ATP are released during cell lysis and blood cell aggregation

*Author for correspondence;
E-mail: christian.rump@ruhr-uni-bochum.de
Current address: Marienhospital Herne, Ruhr-Universität Bochum, Hölkeskampring 40, 44625 Herne, Germany

(Schulze-Lohoff *et al*., 1996). There is some evidence that ATP increases DNA synthesis (Schulze-Lohoff *et al*., 1992), cell proliferation and in addition cell death (Schulze-Lohoff *et al*., 1998; Harada *et al*., 2000).

ATP can stimulate a variety of receptor subtypes. ATP- (P2) Receptors are divided into two major families, the ion channel coupled P2X and the G-protein coupled P2Y-receptors. Beside their intracellular signal transduction mechanisms there are differences in molecular structures and pharmacological profiles. At least, seven members of the P2X family ($P2X_{1-7}$) and seven subtypes of the mammalian P2Y receptors ($P2Y_{1,2,4,6,11,12,13}$) have been cloned (Ralevic & Burnstock, 1998; Communi *et al*., 2001; Hollopeter *et al*., 2001).

The aim of the study was to investigate mitogenic effects of extracellular nucleotides in human mesangial cells. Possible receptor subtypes and intracellular signalling pathways were analysed.

Methods

The present study was approved by the local ethics committee. Human kidneys were obtained from patients undergoing nephrectomy for renal cell carcinoma. Only macroscopic intact renal cortex tissue was used for cell preparation. Renal cortical slices were minced, pushed and rinsed through a serial of stainless-steel sieves of different pore sizes of 40, 80 and finally 120 mesh. Glomeruli retained by the 120 mesh sieves were collected in tubes and centrifuged at $1750 \times g$ for 8 min and suspended in RPMI + L-glutamine containing 20% foetal calf serum (FCS) and supplements (nonessential amino acids, Na-Pyruvat, penicillin, streptomycin, HEPES (Biochrom-chrom, Berlin, Germany), insulin-transferrin-selenite (Boehringer, Mannheim, Germany)). Primary cultures and subcultures were maintained at 37°C and 5% CO_2. Plates were left undisturbed for the next 3–6 weeks to facilitate outgrowth of mesangial cells from glomeruli. Then, the cells were trypsinized and passaged. All cells were studied within the first 15 passages.

Cell identification of mesangial cells

Cell purity was assessed by the peroxidase antiperoxidase (PAP) staining from DAKO (Hamburg, Germany) following the manufacturer's manual. Primary antihuman antibodies used were mouse antifactor VIII-related antigen, mouse anticytokeratin, mouse antivimentin, mouse antidesmin, mouse antismooth muscle actin (all from DAKO) and rabbit anti-WT-1 (Santa Cruz, CA, U.S.A.). Secondary antibodies were mouse anti-rabbit IgG and rabbit anti-mouse IgG (both 1 : 100, DAKO). Mesangial cells were factor VIII and WT-1 negative, showed weak signals for desmin and smooth-muscle actin and strong signals for cytokeratin and vimentin.

Proliferation assays

All experiments were done under growth-arrested conditions. Mesangial cells were plated at a density of 30,000 cells per well in 24-well plates and grown to 80% confluence in RPMI, 20% FCS and supplements before they were placed in growth-arrest medium (RPMI medium supplemented with 0.5% FCS; 5 μg insulin ml^{-1}, Sigma, Deisendorf, Germany; and 100 U penicillin and 100 μg streptomycin ml^{-1}) for 72 h to decrease proliferation.

Determination of DNA synthesis

DNA synthesis was assessed by measuring [^{3}H]thymidine incorporation. All studied substances were added to resting cells (four wells for each concentration) for 24 h. The cells were incubated with [^{3}H]thymidine (1 μCi ml^{-1}) during the last 6 h. The medium was then aspirated, the cells washed three times with phosphate buffer and for DNA precipitation twice with ice-cold 10% trichloroacetic acid (TCA). The fixed cellular material was solubilized in 1 ml 0.5 M NaOH for 2 h mixed with 10 ml scintillation fluid (Ultima-Gold, Canberra Packard, Frankfurt, Germany) to measure the amount of radioactivity present in the TCA insoluble fraction. Data are expressed as the mean ratio of radioactivity present in four wells with identical concentration divided by control (0.5% FCS) values (% of control).

RNA extraction and RT–PCR of P2X and P2Y-receptors

RNA was gained from 2×10^6 cells using the RNAeasy Kit (Qiagen, Hilden, Germany). Following DNA digestion (Rnase free Dnase, Invitrogen, Gibco-BRL, Karlsruhe, Germany) 2 μg of RNA was used in the superscript first-strand system (Gibco-BRL) for synthesis of cDNA. The amplification was performed with specific primer (Table 1) in a volume of 50 μl with 10% of the first-strand cDNA with AmpliTaq Gold (Applied Biosystems, Weiterstadt, Germany) according to the supplier's protocol. After 5 min, 95°C followed by 35–40 cycles consisting of 1 min, 95°C, 1 min 55–65°C, 1 min, 72°C and for termination 8 min 72°C, 10 μl of the reaction products were analysed on a 1.5% agarose gel stained with ethidium bromide.

Determination of $MAPK^{42/44}$ activation

Kinetics of $MAPK^{42/44}$ phosphorylation were done by stimulation of resting mesangial cells plated on six-well plates with ATP (100 μM) for 0, 2, 5, 10, 15, 20 and 60 min. To determinate concentration dependency, various concentrations of ATP, α-β-methylene-ATP and UTP were applied on the cells for 10 min. For experiments using the mitogen-activated protein (MAP) kinase (MEK) inhibitor PD-98059 (New England BioLabs, Beverly, U.S.A.), cells were incubated for 30 min at 37°C in growth-arresting medium that contained the inhibitor before the addition of agonists. After treatment, media were aspirated, solubilized in 200 μl 2 × Laemmli sample buffer with 200 mM dithiothreitol, and boiled for 5 min. Lysates were sonicated to disrupt DNA, and proteins were separated on 10% SDS–PAGE gels. The proteins were electrophoretically transferred to nitrocellulose in buffer containing 25 mM Tris, 192 mM glycine, 20% methanol and 0.02% SDS. The nitrocellulose was blocked with 5% nonfat dry milk in 20 mM Tris, pH 7.4, 150 mM NaCl, and 0.01% Tween-20. The membranes were probed either with a polyclonal phosphotyrosyl-MAP kinase-specific antibody (New England BioLabs) which recognizes only the tyrosine-phosphorylated (active) form of p44 MAP kinase (ERK1) and p42 MAP kinase (ERK2) or with a MAP kinase-specific

Table 1 Sequence, predicted product size and source of primers used for RT–PCR

Primer		*Sequence*	*Product size*	*Source**
$P2Y_1$	Sense:	5′-TGC CAG CCC TGA TCT TCT ACT ACT-3′		
	Antisense:	5′-ATA CGT GGC ATA AAC CCT GTC ATT-3′	608 bp	NM_002563
$P2Y_2$	Sense:	5′-GCC GGG GCC GTG TGG GTG TT-3′		
	Antisense:	5′-CGG GTG ACG TGG AAT GGC AGG AA-3′	335 bp	NM_176072
$P2Y_4$	Sense:	5′-GGG ATG CAA CGG CCA CCT ACA-3′		
	Antisense:	5′-GCA CGA AGC AGA CAG CAA AGA CAG-3′	579 bp	NM_002565
$P2Y_6$	Sense:	5′-CCC TGC TGG CCT GCT ACT GTC TCC-3′		
	Antisense:	5′-TTC TCC GCA TGG TTT GGG GTT GGT-3′	452 bp	U52464
$P2Y_{11}$	Sense:	5′-CCC CCG CTG GCC GCC TAC CTC TTA-3′		
	Antisense:	5′-GCC CAA CCC CGC CAG CAC CAG-3′	393 bp	AF030335
$P2Y_{12}$	Sense:	5′-CTC TGT TGT CAT CTG GGC ATT CAT-3′		
	Antisense:	5′-GGT TTG GCT CAG GGT GTA AGG A-3′	361 bp	AF313449
$P2Y_{13}$	Sense:	5′-TGT GTC GTT TTT CTT CGG TG-3′		
	Antisense:	5′-CTG CCA AAA AGA GAG TTG-3′	575 bp	NM_176894 (Communi *et al*., 2001)
$P2X_1$	Sense:	5′-CTT TCC ACG CTT CAA GGT CAA CA-3′		
	Antisense:	5′-GCC ACC CCA AAG ATG CCA AT-3′	453 bp	NM_002558 (Lynch *et al*., 1999)
$P2X_{2a\text{-}d}$	Sense:	5′-TTT ATC GTG GAG AAG GCT GGG GAG-3′		
	Antisense:	5′-TTT CGT GGA GAT GCT CCG CTA CTG-3′	600–879 bp	AF190822-5
$P2X_3$	Sense:	5′-TCT GTG CTC CGG ACC TGT GAG AT-3′		
	Antisense:	5′-AAG CGG ATG CCA AAA GCC TTC A-3′	491 bp	AB016608
$P2X_4$	Sense:	5′-CCT TCT GCC CCA TAT TCC GTC T-3′		
	Antisense:	5′-GTT GAT CAT AGT GGG GAT GAT GTC A-3′	341 bp	AF191093
$P2X_{5a/b}$	Sense:	5′-GAG TGC TGT CAT CAC CAA AGT CAA-3′		
	Antisense:	5′-CCA GTC GGA AGA TGG GGC AGT A-3′	512/440 bp	U49395
$P2X_6$	Sense:	5′-CCT GTG AGA TCT GGA GTT GGT GC-3′		
	Antisense:	5′-GTG TCC AGG TCA CAA TCC CAG T-3′	319 bp	AF065385
$P2X_7$	Sense:	5′-CGA CTT CCC CGG CCA CAA CTA-3′		
	Antisense:	5′-TGC CAA AAA CCA GGA TGT CAA AAC-3′	383 bp	NM_002562
β-actin	Sense:	5′-ACC TTC AAC ACC CCA GCC ATG TAC G-3′		
	Antisense:	5′-CTG ATC CAC ATC TGC TGG AAG GTG G-3′	645 bp	V00481

*Sources are described by the accession number in GenBank.

antibody (New England BioLabs) which recognizes the total amount of ERK1 and ERK2.

Accordingly specific antibodies raised against phosphorylated and total p38 MAP kinase SAPK/JNK MAP kinase (New England BioLabs) were used. The buffer used for incubation contained 20 mM Tris, pH 7.4, 150 mM NaCl, 3% BSA, and 0.01% Tween-20. The blots were then washed in 20 mM Tris, pH 7.4, 150 mM NaCl and 0.01% Tween-20, and bound antibody was detected by a horseradish peroxidase-conjugated anti-rabbit IgG and enhanced chemiluminescence (Pierce, Rockford, IL, U.S.A). The detection and estimation of the ratio between activated and total protein was performed with the FluorChem (Alpha Innotech Corporation, U.S.A.) Imaging System. The density ratio was used as the quantum of MAPK activation.

Cell number

The number of viable cells was determined by a colorimetric method (CellTiter 96®AQ_{ueous}, Promega, Mannheim, Germany) first described by Cory and co-workers (Cory *et al*., 1991). In brief, a MTS tetrazolium compound is bioreduced by cells into a coloured formazan product that can be measured by absorbance at 490 nm in a plate reader.

The cells were plated into a 96-well plate. After reaching 70% confluence in growth medium and another 2 days in growth-arrest medium the cells were stimulated with the tested substances (ATP, PDGF) for 24 h. The cells were incubated with 20 μl of CellTiter substrate at 37°C in 5% CO_2. After 1 h the absorbance was read at 490 nm. By using standard curves the actual cell number was estimated.

Statistics

All data are expressed as means ± s.e.m. Multiple comparisons with single control were analysed by ANOVA with a *post-hoc* test by Dunnetts. To determine a rank order of potency on DNA synthesis, concentration–response curves were compared by ANOVA (> indicating significant higher potency). Differences in two groups were tested by Student's *t*-test.

Values of $P<0.05$ were considered statistically significant. All data were analysed by SPSS 11.0 (Sigma-Plot, U.S.A.).

Results

Nucleotide-induced [^{3}H]thymidine incorporation in human mesangial cells

In control experiments [^{3}H]thymidine incorporation into mesangial cells was measured in growth-arrest medium containing for 24 h 0.5% FCS but no further drugs. The mean value was set to 100% (control, 0.5% FCS). To test the effect of various drugs, different concentrations were added to the growth-arrest medium for 24 h. ATP, ADP, ATP-γ-S, UTP and ADP-β-S concentration dependently increased [^{3}H]thymidine uptake (Figure 1). 2-m-thio ADP had a similar potency to ADP-β-S (data not shown). UDP and α-β-methylene-ATP failed to induce an effect on [^{3}H]thymidine incorporation (Figure 1).

[^{3}H]thymidine uptake induced by ATP (10 μM) was significantly reduced by the non-subtype selective P2-receptor antagonist suramin (30 μM) from 176$\pm$13% ($n=31$) to 61$\pm$4% ($n=31$) of control (FCS 0.5%) ($P<0.05$).

Involvement of the MAPK pathway

In human mesangial cells ATP (100 μM) induced a fast activation of the MAPK$^{42/44}$ pathway (Figure 2a and b). The maximal activation of MAPK$^{42/44}$ was observed 10 min after addition of ATP. A more than two-fold increase was still present 20 min after incubation with ATP (100 μM). ATP and UTP (10 min) concentration dependently increased MAPK$^{42/44}$ activity (Figure 3). α-β-methylene-ATP and UDP failed to activate MAPK$^{42/44}$ (Figure 3).

The selective MAPK$^{42/44}$ blocker PD 98059 (100 μM) abolished MAPK$^{42/44}$ activation induced by 2 and 5 min 10 μM ATP (157$\pm$10 and 217$\pm$28% of control) to 82$\pm$17 and 94$\pm$16% of control ($n=3$), respectively (activation of MAPK$^{42/44}$ at 0 min in cells treated with PD 98059 100 μM was set to 100%). PD 98059 (100 μM) alone decreased MAPK$^{42/44}$ activity by 84$\pm$19%, compared to values of unstimulated, resting cells ($n=3$).

In parallel, basal and ATP (10 μM) induced [^{3}H]thymidine incorporation was reduced by PD 98059 in a concentration of 10 and 100 μM significantly (Figure 4). Stimulation with ATP (10 μM) did not alter [^{3}H]thymidine uptake in cells treated with 100 μM PD 98059 when compared with cells treated with PD 98059 in the presence of FCS 0.5%. In contrast to that, application of 20% serum did increase [^{3}H]thymidine uptake significantly, suggesting activation of MAPK$^{42/44}$ independent pathways.

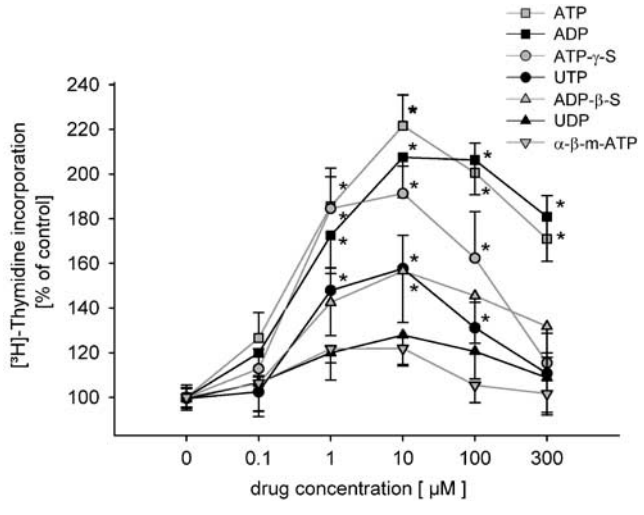

Figure 1 Dose–response curves of various nucleotides on [^{3}H]thymidine incorporation as a marker for DNA synthesis are shown. Medium containing 0.5% FCS was used as a control and set to 100%. Each concentration represents $n=10-85$ data points. *Indicates significant difference between 0.5% FCS (control) and stimulation (ANOVA with *post-hoc* test by Dunnet's).

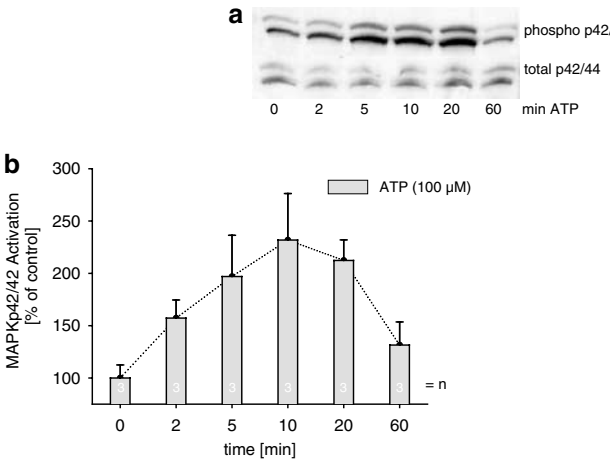

Figure 2 (a) Representative Western blot of ATP (10 μM) induced activation of MAPK$^{42/44}$. Antibody recognizing only the phosphorylated form of MAPK$^{42/44}$ (upper blot) or total MAPK$^{42/44}$ (lower blot) was used. (b) The ratio phosphorylated to total MAPK$^{42/44}$ of three individual preparations was used to demonstrate MAPK$^{42/44}$ activation. The ratio at minute 0 was used as a control and set to 100%. Maximal MAPK$^{42/44}$ activation is present 10 min after adding ATP (100 μM). The number in the column [n] equals the number of experiments.

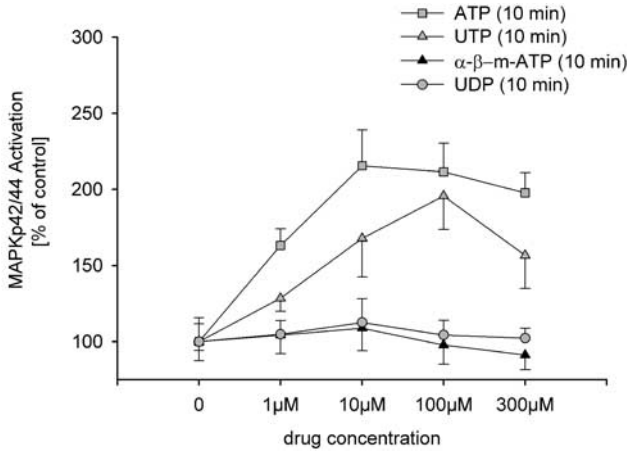

Figure 3 The ratios of phosphorylated to total MAPK$^{42/44}$ of three individual preparations demonstrate concentration-dependent activation of MAPK$^{42/44}$ after 10 min ATP or UTP. α-β-Methylene-ATP and UTP failed to stimulate MAPK$^{42/44}$. The ratio after 10 min without agonists was used as a control and set to 100%.

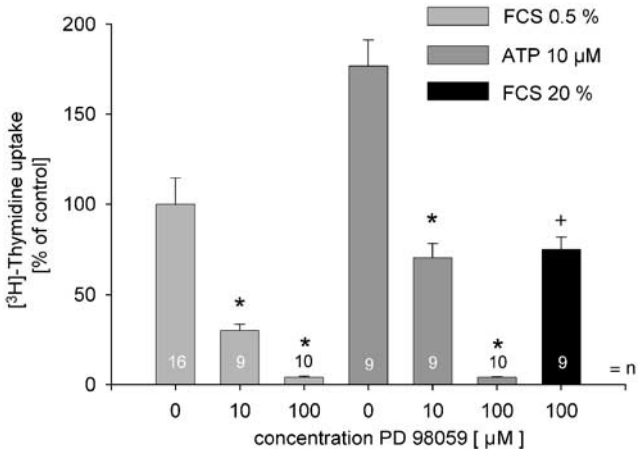

Figure 4 The selective MAPK$^{42/44}$ pathway inhibitor PD 98059 reduced basal (FCS 0.5%) and ATP (10 μM) increased [^{3}H]thymidine incorporation in a concentration-dependent manner. FCS 20% but not ATP (10 μM) altered [^{3}H]thymidine uptake in cells treated with 100 μM PD 98059. The number in the column [*n*] equals the number of experiments. *Indicates significant difference between 0.5% FCS (control) and 10 or 100 μM PD 98059 (ANOVA with *post-hoc* test by Dunnet's). $^+$Indicates significant difference between 100 μM PD 98059 with and without FCS 20% (Student's *t*-test).

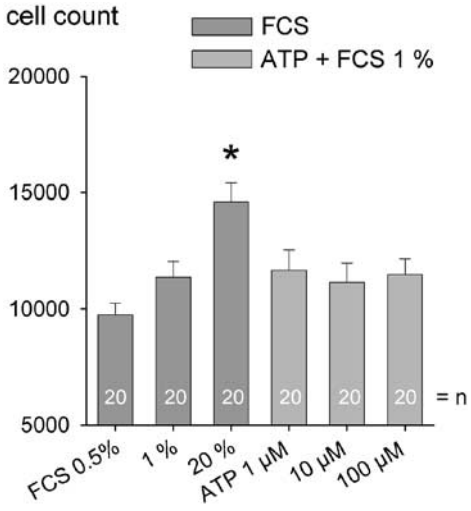

Figure 5 Cell number increased 2 days after incubation with FCS 1% and FCS 20% compared to FCS 0.5%. No additive effects were observed when ATP (1–100 μM) was given on top of FCS 1%. The number in the column [*n*] equals the number of experiments. *Indicates significant difference between 0.5% FCS (control) and FCS 1% and 20% (ANOVA with *post-hoc* test by Dunnet's).

Besides the effects on the MAPK$^{42/44}$ pathway, ATP (1–100 μM) failed to activate MAPK^{jnk} or MAPK^{p38} in human mesangial cells (data not shown).

ATP amplifies growth factor induced cell proliferation

Mesangial cells were plated into a 96-well plate. After reaching 70% confluence, cells were left for 24 h in growth-arrest medium. FCS (1 and 20%), added to the medium, increased cell number in a concentration-dependent manner (Figure 5). ATP (1–100 μM) added in addition to FCS 1% did not further increase the cell number (Figure 5). Platelet-derived growth factor (PDGF; 1 and 5 ng ml^{-1}) also increased the cell number in a concentration-dependent manner (Figure 6). ATP (10 μM) on top of PDGF, amplified PDGF-induced cell proliferation in a synergistic manner (Figure 6). ATP (10 μM) alone had no effect.

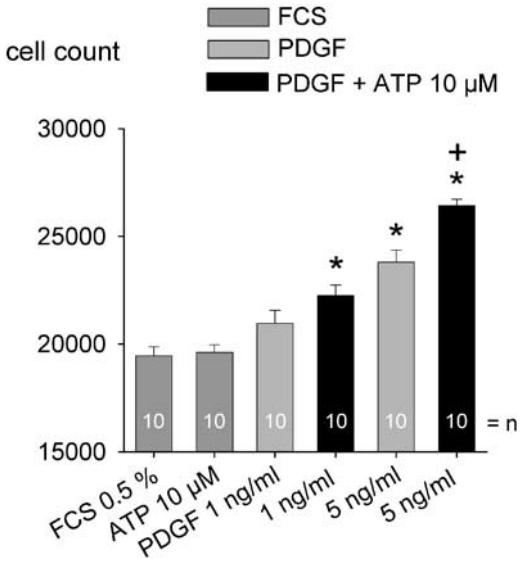

Figure 6 Cell number increased 2 days after incubation with 1 and 5 ng PDGF ml^{-1} (light-grey column) as compared to FCS 0.5%. ATP (10 μM) alone had no effects on top of 0.5% FCS, but amplified PDGF-induced cell proliferation. The number in the column [*n*] equals the number of experiments. *Indicates significant difference between 0.5% FCS (control) and stimulation (ANOVA with *post-hoc* test by Dunnet's). $^+$Indicates significant difference between PDGF (5 ng ml^{-1}) and ATP (10 μM) in combination with PDGF (5 ng ml^{-1}) (Student's *t*-test).

RT–PCR analysis of P2Y- and P2X-receptor mRNA

Resting cells were harvested and the RNA extracted. Under these conditions RT–PCR revealed products of the expected lengths for $P2Y_{1,2,4,6,11,12}$- and $P2X_{1,2,4,5,6,7}$-receptors in human mesangial cells (Figure 7). No expression of $P2X_3$ and $P2Y_{13}$ could be detected. The housekeeper β-actin was used as a positive and negative control. Experiments without reverse transcriptase (–) confirmed that the PCR products originated from mRNA but not from genomic DNA.

Discussion

Overactivity of the sympathetic nervous system is a hallmark of various renal diseases (Converse *et al*., 1992). Moreover, it has been shown that the sympathetic nervous system plays an important role for progression of glomerulosclerosis in an experimental model of chronic renal failure. In this model, an increased release of noradrenaline from renal cortex has been observed (Amann *et al*., 2000). Noradrenaline, however, is not the only neurotransmitter of the sympathetic nervous system. For example, in human and rat kidney cortex neuronal release of the sympathetic cotransmitter ATP has been demonstrated (Rump *et al*., 2000; Vonend *et al*., 2002). In the present study, the possibility that extracellular ATP mediates human glomerular cell proliferation was tested.

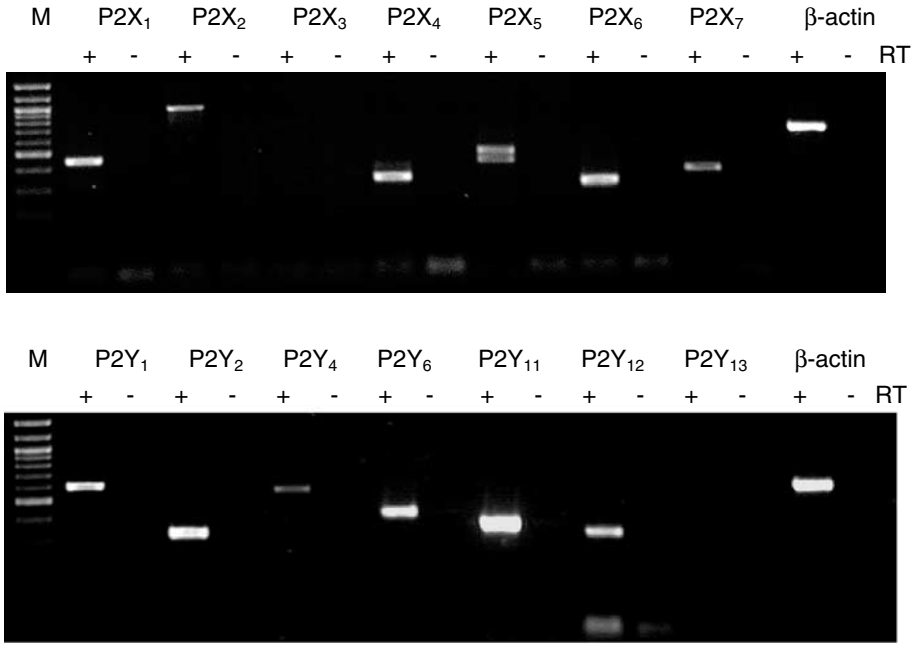

Figure 7 P2Y- and P2X-receptor subtype expression in human cultured mesangial cells. PCR with (+) and without reverse transcriptase (−) and specific primers produced amplification products for $P2Y_{1,2,4,6,11,12}$ and $P2X_{1,2,4,5,6,7}$ at the expected size. Marker is a 100 bp ladder. PCR without RT (−) showed no amplification products.

DNA synthesis is the first important step of the cell cycle towards proliferation. It was shown that ATP increases [^{3}H]thymidine incorporation, as a marker for DNA synthesis. ATP was the most potent P2-receptor agonist used increasing DNA synthesis by more than 200% as compared to control. Comparable results were reported by others using rat mesangial cells in culture (Schulze-Lohoff *et al*., 1992; Huwiler & Pfeilschifter, 1994; Harada *et al*., 2000).

The next step was to evaluate the effects of ATP on cell number as a marker for hyperplasia. Increasing effects of ATP on the cell number have been shown in experiments on rat mesangial (Schulze-Lohoff *et al*., 1992; 1995) and smooth muscle cells (Wang *et al*., 1992; Erlinge *et al*., 1993). In our experiments on human mesangial cells, ATP given in addition to PDGF increased the cell number whereas no increasing effects were observed in the presence of FCS alone (i.e. without the addition of PDGF). Thus, in some cell types such as the human mesangial cells the growth promoting effect of ATP seems to require the presence of other growth factors. Since ATP by itself did not increase cell number, ATP and PDGF seem to have synergistic effects as previously shown in cultured smooth muscle cells (Crowley *et al*., 1994; Erlinge, 1998). A possible explanation for the described dissociation of DNA synthesis and increase of cell number by ATP was put forward by Schulze-Lohoff and co-workers. He suggested that ATP stimulates cells to proceed into the S phase but fails to promote further steps essential for cell division (Schulze-Lohoff *et al*., 1995).

To further prove the role of ATP as an extracellular signalling molecule involved in cell proliferation, we investigated whether ATP activates the MAPK signalling cascades as one of the most important links between receptor activation and cellular responses. Different MAPK pathways are associated with cell proliferation, cell differentiation, cell movement and cell death. These are the $MAPK^{42/44}$ and the stress-induced $MAPK^{jnk}$ and $MAPK^{p38}$ cascades, which can be activated by various substances including cytokines, growth factors and neurotransmitters. In the present study, we investigate whether ATP-triggered proliferation involves phosphorylation of $MAPK^{42/44}$, $MAPK^{jnk}$ and $MAPK^{p38}$. ATP induced a time- and concentration-dependent activation only of the $MAPK^{42/44}$ pathway. A coupling to this pathway was confirmed by the blockade of the effects of ATP on $MAPK^{42/44}$ phosphorylation and in parallel on DNA synthesis by the selective MEK inhibitor PD 98059. Since the $MAPK^{42/44}$ pathway is shared by a variety of growth factors, blockade by PD 98059 leads to reduction in DNA synthesis even in cells not stimulated by ATP. ATP also activates $MAPK^{42/44}$ activity in rat mesangial cells (Huwiler & Pfeilschifter, 1994), glioma (Tu *et al*., 2000), PC12 (Soltoff *et al*., 1998; Swanson *et al*., 1998) cell lines and vascular smooth muscle cells (Wilden *et al*., 1998).

In contrast to observations in rat mesangial cells (Huwiler *et al*., 1997; 2000) we could not demonstrate activation of $MAPK^{jnk}$ and $MAPK^{p38}$ in human mesangial cells. This suggests that there are important differences between species in P2-receptor-mediated signalling processes involved in ATP-mediated glomerular cell proliferation.

Interestingly, the dose–response curves of ATP-analogue on DNA synthesis revealed, that after reaching the maximum, a decrease in synthesis can be obtained by using higher concentrations. At present no plausible explanation for this observation can be given. Further studies whether the cytolytic $P2X_7$ receptor might here be involved, activating antiproliferative pathways as postulated for rat mesangial cells (Schulze-Lohoff *et al*., 1998; Harada *et al*., 2000) are needed and are currently under investigation in our laboratory.

It is well known that many cell types express more than one P2-receptor (Ralevic & Burnstock, 1998). Accordingly, in the present study, the RT–PCR analysis demonstrated

$P2Y_{1,2,4,6,11,12}$ and $P2X_{1,2,4,5,6,7}$ mRNA in human mesangial cells. Therefore, the question arises which P2-receptor subtype mediates the observed mitogenic effects of ATP. Generally, the rank order of potency of nucleotides on DNA synthesis was ATP ≈ ADP > ATP-γ-S > UTP ≈ ADP-β-S ≈ 2-m-thio-ADP > UDP ≈ α-β-methylene-ATP.

This profile and the observed antagonism of suramin excludes an involvement of the α-β-methylene-ATP-sensitive $P2X_1$- and $P2X_3$- and suramin-insensitive $P2X_4$-, $P2X_6$- and $P2X_7$-receptor subtypes in the effects induced by ATP (Khakh *et al*., 2001; Lambrecht *et al*., 2002). Since our RT–PCR data suggest the presence of α-β-methylene-ATP-insensitive, suramin-sensitive $P2X_2$ and $P2X_5$ receptors in human mesangial cells, an involvement of these receptor subtypes in DNA synthesis cannot be ruled out. However, ADP and UTP, which both do not activate $P2X_2$ and $P2X_5$ receptors, significantly increased [^{3}H]thymidine uptake. Therefore, it seems to be convincing that P2Y-receptors play a major role as a comitogen in human mesangial cells. This is in contrast to observations on PC12- (Swanson *et al*., 1998) or MG-63-cells (Nakamura *et al*., 2000), where ATP but not ADP or UTP induced MAPK activation and DNA synthesis.

The effectiveness of ATP, ADP, ATP-γ-S, ADP-β-S and 2-methylthio-ADP is in agreement with an involvement of $P2Y_1$-, $P2Y_{11}$-, $P2Y_{12}$- and $P2Y_{13}$- receptors (Ralevic & Burnstock, 1998; von Kugelgen & Wetter, 2000; Communi *et al*., 2001). The $P2Y_{13}$-receptor does not seem to play a major role in human mesangial cells since RT–PCR could not detect mRNA expression. Suramin (30 μM) reduced the effect on DNA synthesis. Because the $P2Y_{12}$-receptor subtype is only weakly antagonized by suramin (Unterberger *et al*., 2002), a major involvement of this receptor is also less likely. In contrast to that suramin, given at a relatively low concentration of 30 μM, can antagonize $P2Y_1$- and $P2Y_{11}$-receptors (von Kugelgen & Wetter, 2000), suggesting their involvement in modulating proliferative effects in human mesangial cells. However, in haematological cells transfected with human $P2_{Y11}$ receptors, α-β-methylene-ATP was able to activate this receptor subtype (van der Weyden *et al*., 2000). As no response to α-β-methylene-ATP could be observed in our study, one could argue against major involvement of this receptor subtype. The effect of UTP indicates the additional presence of an UTP-sensitive $P2Y_2$- or $P2Y_4$-receptor (Ralevic & Burnstock, 1998; von Kugelgen & Wetter, 2000). $P2Y_6$-receptors does not seem to have an effects on $MAPK^{42/44}$ activation and DNA synthesis as shown by the lack of efficacy of the $P2Y_6$-agonist UDP (Ralevic & Burnstock, 1998; von Kugelgen & Wetter, 2000). The mitogenic effect of UTP is in clear contrast to observations in another human renal cell line (Vonend *et al*., 2002). Although ATP had a strong effect on DNA synthesis in human podocytes comparable to that in human mesangial cells, UTP was without an effect (Vonend *et al*., 2002). This underlines functional diversities of P2Y-receptors even within one organ and species.

Subtype-selective ligands are still lacking for most of the human P2-receptor subtypes. However, the presented data suggest a role of the adenine nucleotide sensitive $P2Y_1$-receptors on the one hand and of the uracil nucleotide sensitive $P2Y_2$- or $P2Y_4$-receptors on the other hand on cell proliferation in human mesangial cells. Nevertheless, a supplementary role of other subtypes, like $P2X_2$- or $P2X_5$-receptors cannot be excluded when the endogenous agonist ATP is present.

In conclusion, we demonstrated the ability of the extracellular signal molecule ATP to trigger proliferation in cultured human mesangial cells. P2Y-receptor stimulation activates the Ras, Raf, $MAPK^{42/44}$ signal transduction pathway, increases DNA synthesis and leads to amplification of growth factor induced cell proliferation. Therefore, ATP release by cell stress or sympathetic overactivity has the potential to play a major role in the progression of chronic renal failure by accumulating extracellular matrix. Further studies with subtype selective antagonists or P2-receptor subtype overexpression are needed for further classification of P2-receptors in human mesangial cells. Experiments with subtype selective antagonists will also facilitate the understanding of the role of ATP in maladaptive changes observed in a variety of renal diseases.

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