Nephrol Dial Transplant (2006) 1 of 10
doi:10.1093/ndt/gfl589

NDT
Nephrology Dialysis Transplantation

Original Article

Increased expression of the pro-apoptotic ATP-sensitive $P2X_7$ receptor in experimental and human glomerulonephritis

Clare M. Turner[1], Frederick W. K. Tam[3], Ping-Chin Lai[3,5], Ruth M. Tarzi[3], G. Burnstock[2], Charles D. Pusey[3], H. Terence Cook[4] and Robert J. Unwin[1]

[1]Department of Physiology and [2]Autonomic Neuroscience Institute, University College London (Hampstead Campus), [3]Renal Section, Division of Medicine and [4]Department of Histopathology, Imperial College, London, UK and [5]Kidney Institute, Chang Gung Memorial Hospital, Taiwan

Abstract

Background. The involvement of IL-1β and other pro-inflammatory cytokines in most forms of glomerulonephritis is now well established. The $P2X_7$ receptor, an ATP-sensitive P2X receptor, functions not only as a non-selective cation channel, but it is also involved in the rapid processing and release of IL-1β, apoptosis and necrotic cell death. Therefore, we wanted to investigate if expression of this receptor is altered in the glomeruli of rodent models of glomerulonephritis.

Methods. $P2X_7$ receptor protein expression was investigated using immunohistochemistry, and apoptosis was assessed using the TUNEL assay and caspase-3 immunostaining. Real-time PCR with gene-specific primers was used to detect $P2X_7$, IL-1β, p53, bax and bcl-2 mRNA expression.

Results. Although the levels of the $P2X_7$ receptor protein in mouse kidney are normally very low, or undetectable, we detected an increase in glomerular expression of this receptor and an increase in glomerular apoptotic cells in a mouse model of accelerated nephrotoxic nephritis. We also observed increased glomerular and tubular expression of the $P2X_7$ receptor protein in renal biopsy tissue of patients with autoimmune-related glomerulonephritis. Furthermore, $P2X_7$ receptor mRNA increased in the kidneys of a rat model of proliferative glomerulonephritis and this coincided with the onset of proteinuria. We also observed increased mRNA expression of Il-1β and the pro-apoptotic markers p53 and bax, but not of anti-apoptotic bcl-2.

Conclusion. Although there is an association between expression of the pro-inflammatory and pro-apoptotic $P2X_7$ receptor and glomerulonephritis in these rodent models, and in at least one form of human glomerulonephritis, the underlying relationship and its functional significance remain to be explored.

Keywords: apoptosis; ATP; glomerulonephritis; $P2X_7$ receptor

Introduction

Glomerulonephritis (GN) is still a leading cause of end-stage renal disease and its treatment is non-specific immunosuppression, which has significant adverse side effects. Deposition of antibodies against autoantigens, or exogenous antigens, leading to immune complex-mediated inflammation and tissue injury has been well documented in clinical disease and in experimental models of GN [1].

Multiple cytokines are involved in the pathogenesis of GN via recruitment of monocytes, macrophages and T cells into glomeruli [2,3]. Moreover, increased glomerular production of interleukin-1β (IL-1β), tumour necrosis factor-α (TNF-α) and monocyte chemo-attractant protein-1 (MCP-1) has been detected in rodent models of experimental GN [4,5], and glomerular IL-1β has been found in patients with proliferative forms of GN [6,7]. Treatment with IL-1 receptor antagonist, or TNF-α inhibitor, has been shown to suppress the development of rat crescentic anti-glomerular basement membrane (GBM) GN [8,9].

ATP, via the $P2X_7$ receptor, has been shown to rapidly promote the processing and release of mature IL-1β from lipopolysaccharide (LPS) primed macrophages and monocytes [10–14]. Indeed, $P2X_7$ receptor knockout mice fail to generate mature IL-1β from macrophages challenged with LPS and ATP [15], and exhibit a reduced inflammatory response to antibody-induced arthritis [16]. It has also been reported that a $P2X_7$ receptor agonist, 3′-O-(4-benzoyl) benzoyl

Correspondence and offprint requests to: Dr Clare Turner, Epithelial Transport and Cell Biology Group, Department of Physiology, University College London (Hampstead Campus), Rowland Hill Street, London NW3 2PF, UK. Email: c.turner@medsch.ucl.ac.uk

ATP (BzATP), can induce TNF-α production in rat and human microglia [14,17], as well as expression of MCP-1 in cultured rat astrocytes [18].

The $P2X_7$ receptor is a member of the P2X receptor family of ligand-gated cation channels, which comprises seven subtypes, $P2X_{1–7}$ [19]. All seven members of the P2X receptor family form non-specific cation channels on binding to a nucleotide ligand. Although structurally similar to the other P2X receptors, the $P2X_7$ receptor has several unique properties: its affinity for ATP is low, requiring 10–100 times higher concentrations of ATP for activation compared with other P2X receptors [20,21]; stimulation of the $P2X_7$ receptor after brief exposure to ATP leads to apoptosis in several cell types, including macrophages and lymphocytes [20,22], dendritic cells [23] and mesangial cells [24]; sustained activation triggers formation of a large non-selective pore for molecules up to 900 Da in size [25,26]. The opening of the membrane pore is thought to cause cell death by disrupting ionic gradients and/or providing pathways for efflux of vital intracellular molecules [20].

The $P2X_7$ receptor is expressed in immune cells, including monocytes, macrophages, lymphocytes and bone marrow-derived cells [16,27]. In normal adult rat kidney, there is little or no $P2X_7$ receptor expression [28]; however, increased expression has been observed in the glomeruli of diabetic and hypertensive rat models [29]. A low level of $P2X_7$ receptor expression has also been detected in cultured mesangial cells, which can be upregulated by exposure to TNF-α [24]. Currently, very little is known about expression of the $P2X_7$ receptor in other forms of renal disease, including GN. We have been able to demonstrate increased expression of $P2X_7$ receptor protein in glomeruli of a mouse model of GN, and in renal biopsy tissue from patients with lupus nephritis. There was double-labelling of the $P2X_7$ immunostaining in the mouse model with caspase-3 indicating apoptotic cells. Moreover, we have also shown increased mRNA expression of the $P2X_7$ receptor, IL-1β and of the pro-apoptotic genes p53 and bax in a rat model of proliferative GN.

Methods

Experimental models

The rat model of proliferative and necrotizing GN was used previously [30]. Briefly, male Wistar Kyoto rats weighing 200–250 g were injected with 0.1 ml of rabbit anti-rat GBM globulin and then sacrificed at 2, 4 and 7 days after the nephrotoxic serum injection ($n = 6$ for each time-point). The nephrotoxic serum injection causes a rapid influx of monocytes and macrophages into glomeruli within hours of administration, followed by segmental fibrinoid necrosis by day 4 [30]. This is a well-characterized model with the onset of detectable proteinuria and maximal macrophage infiltration of glomeruli occurring 4 days after the nephrotoxic serum injection [30]. Kidneys were removed for RNA extraction. This model proved unsuitable for immunostaining due to cross-reaction between the anti-rabbit secondary antibody used to detect $P2X_7$ positive staining and the rabbit anti-GBM used to induce GN.

Mouse renal tissue obtained previously [31] was used in the present study. The mouse model is an accelerated form of nephrotoxic nephritis in which C57BL/6 mice (purchased from Harlan Ltd, Bicester, UK) were first pre-immunized with 0.2 mg sheep IgG mixed with Freund's complete adjuvant (50:50 v/v) (Sigma, Poole, UK). Five days later mice were given an injection of 10 mg sheep anti-mouse GBM globulin [31]. The animals were sacrificed at 24 h and 8 days after nephrotoxic serum injection and their kidneys embedded in Tissue-Tek (Sakura Finetek, The Netherlands) in preparation for cryosectioning. This GN model is characterized by leukocyte infiltration, proteinuria, glomerular capillary thrombosis, glomerular crescent formation and renal impairment. All procedures were carried out in accordance with the Animals (Scientific Procedures) Act 1986.

Patients with the clinical features of lupus nephritis (including positive anti-double stranded DNA antibodies and diagnostic renal histology) were selected for this study and paraffin sections from their renal biopsies were examined. The Hammersmith, Queen Charlotte's and Chelsea Hospitals Research Ethics Committee approved the study and all the patients who contributed biopsy samples gave their consent.

Real-time PCR

Glomeruli from the rat model of proliferative GN were isolated from the renal cortex using a differential sieving technique, described in detail in [3]. Total glomerular RNA was extracted using RNAzol B according to the manufacturer's protocol (Biogenesis, Poole, UK). One microgram of glomerular RNA was reverse transcribed with 0.5 μg oligo(-dt) 12–18 primer and a first-strand cDNA synthesis kit, (Superscript II RNase H^- reverse transcriptase, Gibco BRL, UK). The resulting cDNA transcripts of glomerular mRNA were used for PCR amplification using the Roche Lightcycler (Roche diagnostics, Germany) and QuantiTect SYBR® Green PCR kit (Qiagen, West Sussex UK). Gene-specific primers for $P2X_7$ receptor, IL-1β, the apoptosis markers p53, bax and bcl-2 and the constitutively expressed gene hypoxanthine phosphoribosyl transferase (HPRT) were used (Table 1). To quantify mRNA expression, standard curves were generated with known amounts of each gene product. A ratio of relative abundance of each gene to the housekeeping gene HPRT was calculated by the Lightcycler Relative Quantification software version 1.0 (Roche Diagnostics, Germany). Melting curve analysis was carried out to ensure primer specificity. PCR products were also analysed by gel electrophoresis and visualized using a Bio-Rad multi-imager (Bio-Rad, Hemel Hempstead, Herts, UK).

Immunohistochemistry

Mouse kidneys were embedded in Tissue-Tek (Sakura Finetek) and sectioned at 8 μm using a cryostat (Reichert Jung CM1800), collected on gelatin-coated slides and air-dried at room temperature. Slide-mounted sections

Table 1. Gene-specific forward and reverse primer sequences used in the study

Gene		Sequence	Accession
P2X$_7$	F	GTGCCATTCTGACCAGGG TTGTATAAA	NM 019256
	R	GCCACCTCTGTAAAGTTC TCTCCGATT	
IL-1β	F	CACCTCTCAAGCAGAGCACAG	NM_031512
	R	GGGTTCCATGGTGAAGTCAAC	
P53	F	CAGCTTTGAGGTTCGTGTTTGT	NM 030989
	R	ATGCTCTTCTTTTTTGCGGAAA	
bax	F	CCAAGAAGCTGAGCGAGTGTCTC	NM 017059
	R	AGTTGCCATCAGCAAACATGTCA	
Bcl-2	F	GGAGCGTCAACAGGGAGATG	NM 016993
	R	GATGCCGGTTCAGGTACTCAG	
HPRT	F	GCTGACCTGCTGGATTACATTA	NM 012583
	R	CCACTTTCGCTGATGACACAA	

were fixed for 2 min in 4% formaldehyde diluted with phosphate-buffered saline (PBS).

Sections (4 μm) were cut from archived wax-embedded human kidney tissue using a microtome, placed on poly-L-lysine-coated slides and dried overnight at 37°C. The sections were subsequently de-waxed with Histoclear (National Diagnostics, Hessle, UK) and re-hydrated in decreasing concentrations of ethanol. Slides were heated in 10 mM citrate buffer (pH 6.0) in a microwave oven for 10 min and allowed to cool for 1 h.

The procedure for both frozen and wax-embedded sections was as follows: slides were washed three times for 5 min each with excess PBS. Non-specific binding was blocked by incubation with 10% normal horse serum (NHS) (Invitrogen Ltd., Renfrew, UK) for 30 min. Subsequently, the sections were incubated overnight at room temperature with the primary antibody, rabbit anti-P2X$_7$ (Roche Bioscience, Palo Alto, CA) at 5 μg/ml diluted in 10% NHS. The following day, slides were washed three times for 5 min each with excess PBS and the secondary antibody for fluorescent microscopy was Cy3 labelled anti-rabbit IgG (Abcam, Cambridge, UK).

Co-staining experiments

To identify macrophages, slides were incubated for 1 h with a biotin-conjugated anti-CD68 antibody (Serotec Ltd., Oxford, UK), and the secondary antibody was streptavidin-linked FITC (Amersham Lifescience, Bucks, UK).

To identify podocytes, the podocyte nuclear marker WT-1 was used; however, antibodies for P2X$_7$ and WT-1 were raised in the same species and so further steps were required to avoid cross-reactivity. P2X$_7$ receptor immunoreactivity was detected by tyramide signal amplification (Renaissance, TSA indirect, NEN, USA) using antibody concentrations below the detection limit of a fluorophore-coupled secondary antibody. After incubation overnight with anti-P2X$_7$, the layers of secondary antibody were biotinylated donkey anti-rabbit IgG (Jackson Immunoresearch, Luton, UK) and ExtrAvidin peroxidase (Sigma); the tyramide signal amplification solution was applied for 8 min and the final layer was streptavidin-linked FITC (Amersham Lifescience). Finally, sections were incubated overnight with anti-WT-1 and then detected with donkey anti-rabbit Cy3. Controls were performed by incubating slides with pre-immune rabbit IgG and with P2X$_7$ receptor antibody pre-absorbed with the homologous peptide—no staining was observed.

For caspase-3 co-staining experiments, mouse GN slides were prepared as described above. Non-specific binding was blocked by incubation with 10% NHS (Invitrogen Ltd.) for 30 min. Subsequently, the sections were incubated overnight at room temperature with the primary antibody, goat anti-P2X$_7$ (Santa Cruz Biotechnology, Ca, USA) at 1 μg/ml and rabbit anti-caspase 3 (Abcam) at 1 μg/ml diluted in 10% NHS. The following day, slides were washed three times for 5 min each with excess PBS and incubated with Cy3-conjugated anti-goat IgG for 1 h, again washed with PBS three times 5 min each and finally incubated with FITC-conjugated anti-rabbit IgG for 1 h.

Slides were mounted in Citifluor (Citifluor Ltd., London, UK) and examined using a Zeiss Axioplan immunofluorescent microscope (Zeiss, Göttingen, Germany), and photographs were documented using a Leica DC200 digital camera (Leica, München, Germany).

Identification of apoptotic cells by TUNEL assay

The terminal deoxynucleotidyl transferase-mediated nick end-labelling (TUNEL) assay was performed using the *in situ* cell death detection kit (Roche Molecular Biochemicals, Mannheim, Germany). TUNEL identifies fragmented DNA as a marker of apoptosis. Briefly, mouse GN kidney sections were fixed with ice cold acetone for 10 min and then washed three times with PBS. Cells were permeabilized with 0.1% triton-X-100 in PBS for 2 min at 4°C, rinsed three times with PBS and incubated for 1 h in the TUNEL reaction mixture (terminal deoxynucleotidyl transferase with FITC conjugated dUTP). After a further washing in PBS, the slides were mounted in citifluor and examined with a Zeiss Axioplan light/fluorescent microscope, and images were captured using a Leica DC200 digital camera.

Statistics

Real-time PCR results are expressed as means ± SEM of n observations. To compare sets of data, one-way ANOVA, followed by the Tukey–Kramer multiple comparisons *post hoc* test, was used ('Graphpad Instat' version 3.06). Differences were considered statistically significant at $P < 0.05$.

Results

Increased mRNA expression of the P2X$_7$ receptor, IL-1β and pro-apoptotic p53 and bax genes, but not anti-apoptotic bcl-2 in rat GN

Experiments were repeated in triplicate in six rats at each time-point, expression levels were calculated as a ratio to the housekeeping gene HPRT and control values were normalized to 1.0 (Figure 1). Glomerular P2X$_7$ receptor mRNA expression was unchanged on

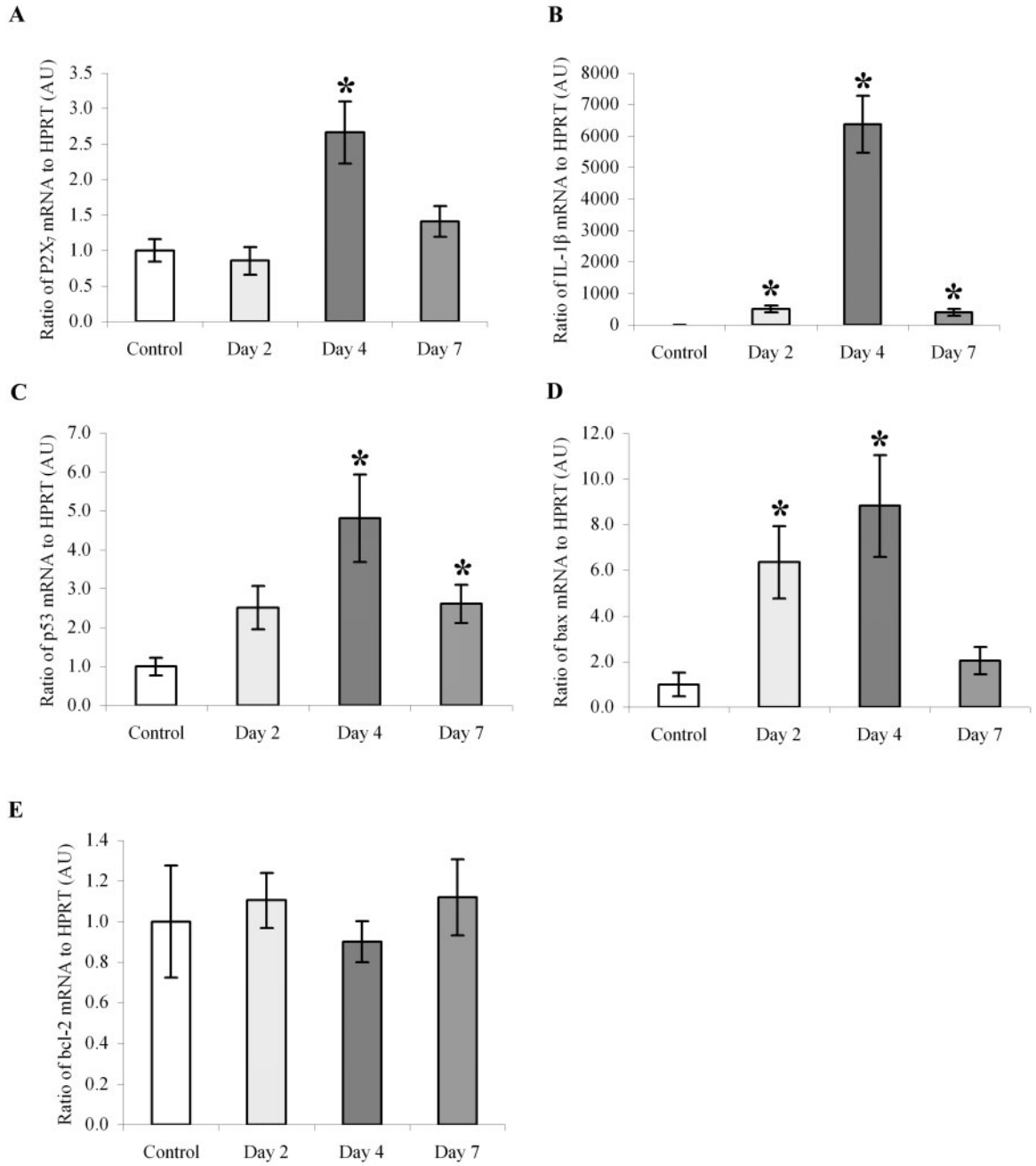

Fig. 1. $P2X_7$ receptor, IL-1β, P53 and bax gene mRNA is upregulated in experimental glomerulonephritis. The expression of mRNA encoding (**A**) $P2X_7$ receptor, (**B**) IL-1β, (**C**) p53, (**D**) bax and (**E**) bcl-2, in kidneys from rats after induction of experimental proliferative glomerulonephritis. Mean expression at each time-point is expressed as a ratio to that of the housekeeping gene HPRT; control (no GN) values are normalized to one ($n = 6$ for each time-point, $^*P \leq 0.01$, error bars = ± SEM).

day 2 after nephrotoxic serum injection, but increased 3-fold by day 4 ($P < 0.01$), which coincided with the onset of detectable proteinuria and the peak in macrophage infiltration reported previously in this model [30]. By day 7, there was no significant increase in $P2X_7$ receptor expression.

Expression of IL-1β mRNA was significantly increased in this model: by day 2, IL-1β had increased 500-fold ($P < 0.001$) and by day 4, this had increased to 6000-fold ($P < 0.001$); by day 7, this had fallen to a 400-fold increase ($P < 0.001$).

Expression of the pro-apoptotic p53 and bax genes increased 2.5-fold (NS) and 6-fold ($P = 0.01$), respectively, by day 2 and was increased maximally by day 4 at 5-fold ($P < 0.01$) and 9-fold ($P < 0.01$), respectively. By day 7, p53 and bax expression levels were still increased, but only 3-fold ($P = 0.01$) and 2-fold (NS), respectively. Expression of the

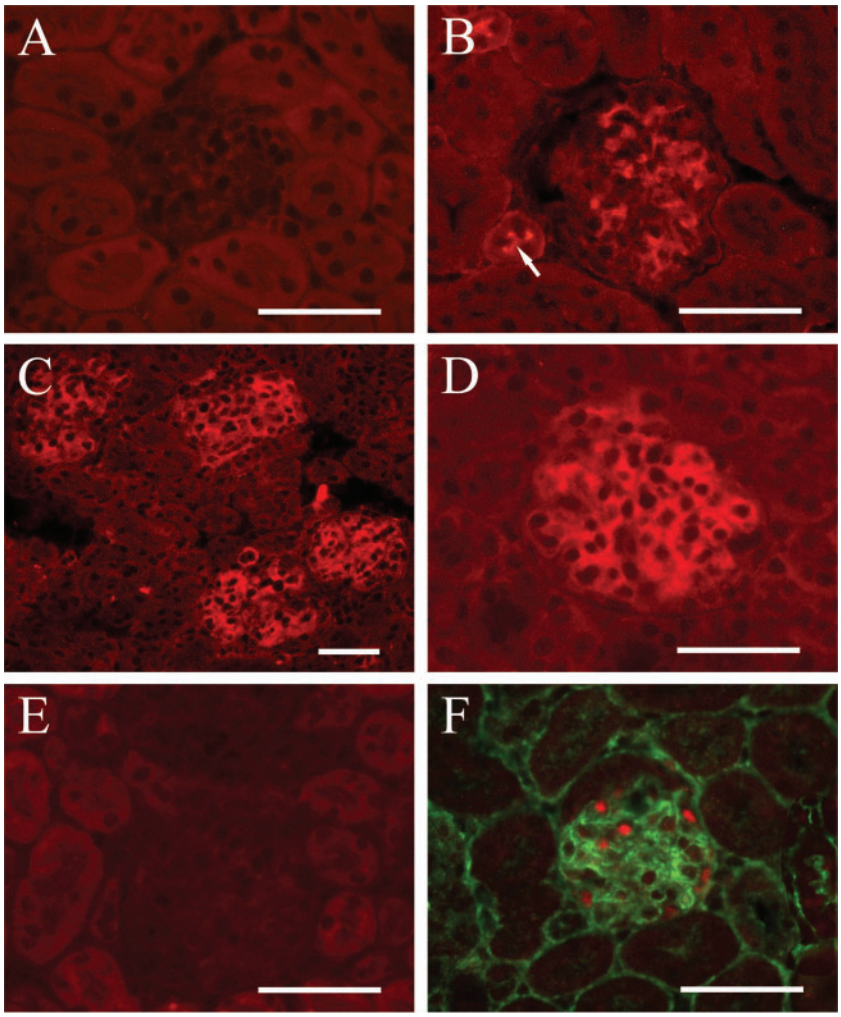

Fig. 2. Increased P2X$_7$ receptor protein expression in mouse accelerated nephrotoxic nephritis compared with control. (**A**) Photomicrograph of a control mouse glomerulus showing minimal P2X$_7$ receptor immunoreactivity (scale bar = 50 μm). (**B**) Photomicrograph of a mouse glomerulus 24 h post-injection of nephrotoxic serum showing dense and localized P2X$_7$ receptor immunoreactivity in glomerular cells and apical expression on distal tubule cells (arrow) (scale bar = 50 μm). (**C**) and (**D**) Mouse glomeruli after 8 days post injection of nephrotoxic serum showing dense P2X$_7$ receptor immunoreactivity (scale bars = 50 μm). (**E**) An immunohistochemical control showing no immunostaining of a mouse glomerulus, 8 days post-injection, P2X$_7$ receptor antibody pre-absorbed with excess homologous peptide (scale bar = 30 μm). (**F**) P2X$_7$ receptor immunoreactivity (green) 24 h post-injection co-stained with the podocyte marker WT-1 (red) showing no co-localization (scale bar = 50 μm).

anti-apoptotic gene bcl-2 was unchanged at each time-point.

Increased glomerular expression of P2X$_7$ receptor protein in mouse GN

P2X$_7$ receptor immunoreactivity was not detectable in normal mouse kidney (Figure 2A). However, P2X$_7$ receptor immunoreactivity was clearly visible in the glomeruli of mice with accelerated nephrotoxic nephritis and was readily detectable 24 h ($n = 5$) and 8 days ($n = 7$) post injection of the nephrotoxic serum (NTS). A pattern suggesting mesangial staining was observed in the glomeruli (Figure 2B–D). Apical membrane distal tubule staining was also evident 24 h post injection (Figure 2B). No immunoreactivity was seen in mice glomeruli, neither with non-immune serum nor when the antibody was pre-absorbed with homologous peptide (Figure 2E). To determine whether podocytes or macrophages were immuno-positive, co-localization studies were carried out. P2X$_7$ receptor immunoreactivity did *not* co-localize with the podocyte nuclear protein WT-1 (Figure 2F). However, P2X$_7$ receptor expression was detected in glomeruli (Figure 3A); infiltrating macrophages were identified with anti-CD68 antibody (Figure 3B). Areas of co-localization appear yellow where P2X$_7$ receptor positive macrophages were identified (Figure 3C) alongside non-macrophage glomerular P2X$_7$ receptor immunostaining.

Increased numbers of apoptotic cells in mouse GN

Active caspase-3 is part of the apoptotic machinery of the cell and has previously been reported to increase with time in experimental glomerulonephritis [32].

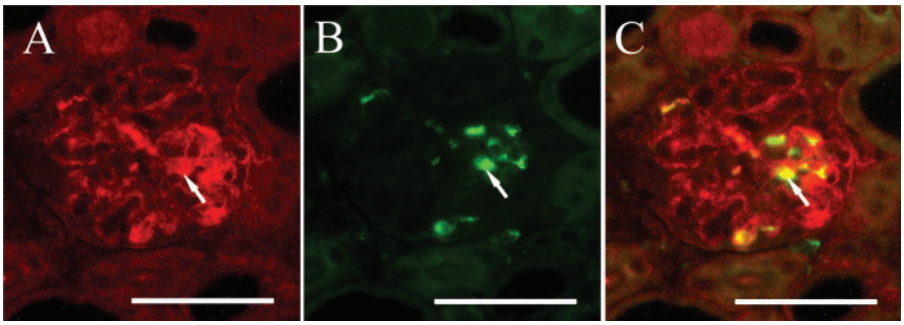

Fig. 3. Expression of $P2X_7$ receptor on glomerular macrophages. Photomicrographs of a mouse glomerulus 8 days post-injection of nephrotoxic serum showing (**A**) $P2X_7$ immunoreactivity and (**B**) CD68 positive macrophages. In the overlay (**C**), areas of co-localization appear yellow, demonstrating $P2X_7$ receptor positive macrophages (arrows) and additional $P2X_7$ receptor staining of glomerular cells (scale bars = 50 μm).

Caspase-3 immunoreactivity was detected in some glomerular cells in mouse GN, 24 h post-injection with NTS and this co-localized with $P2X_7$ immunoreactivity (Figure 4A–C). Moreover, there was an increase in caspase-3 positive cells in glomeruli and some tubular cells 8 days post-NTS and again this co-localized with $P2X_7$ receptor expression (Figure 4D–F). Caspase-3 and $P2X_7$ immunoreactivity could not be detected in normal mouse tissue (Figure 4G–I). TUNEL-positive cells, as an index of cell apoptosis, were also detected in glomeruli and surrounding tubules in mouse GN 8 days post-injection of the NTS (Figure 5A). TUNEL-positive cells were undetectable in normal mouse kidney (Figure 5B).

Altered $P2X_7$ protein expression in lupus nephritis

We also investigated $P2X_7$ receptor protein expression in patients with lupus nephritis: proliferative lupus GN ($n = 6$) and membranous lupus GN ($n = 3$). $P2X_7$ receptor protein expression was detectable in biopsy tissue from all of the patients examined. The majority of the $P2X_7$ immunopositive staining was found in the glomeruli (Figure 6A and B); however, the density of immunostaining varied between glomeruli, even in the same patient (Figure 6C). Dense intracellular staining was found in some proximal tubules and positive staining was detectable in Bowman's capsule (Figure 6C). Apical tubular immunostaining was also detected in some tubules in the outer medulla (Figure 6D). No $P2X_7$ receptor immunoreactivity was observed in GN biopsy tissue when the antibody was pre-absorbed with homologous peptide (Figure 6E) or when pre-immune serum was used.

Control kidney tissue was obtained from four patients: patients undergoing nephrectomy for renal carcinoma ($n = 2$) during which a small portion of normal kidney tissue distant from the tumour site was also excised (to ensure complete tumour excision), or from cadaver donor kidneys removed for renal transplantation, but found to be unsuitable for transplantation ($n = 2$). Very little $P2X_7$ receptor immunoreactivity was detectable in tubules or glomeruli of the human control tissue (Figure 6F). However, $P2X_7$ immunopositive vascular smooth muscle cells were seen in both control tissue and in GN biopsy tissue (not shown).

Discussion

The $P2X_7$ receptor (previously called P_{2Z}) is normally expressed by cells of the immune system, but there is very little expression in normal kidney tissue [20,27,28]. Expression of this receptor has been detected in cultured mesangial cells on exposure to TNF-α [24] and in podocyte cells and renal tubular cells, where expression is upregulated in chronic and inflammatory conditions [29,33]. The present study provides the first reported evidence for increased $P2X_7$ mRNA expression in a rat model of GN and increased expression of $P2X_7$ receptor protein in the glomeruli of a mouse model of accelerated nephrotoxic GN, as well as in patients with GN.

Currently, the role of $P2X_7$ in the etiology of GN is unknown. However, previous studies have shown that brief stimulation of the $P2X_7$ receptor promotes activation of caspase-1 and release of the pro-inflammatory cytokine IL-1β from activated macrophages and HEK-293 cells [13,34,35]. Caspase-1, also known as IL-1β-converting enzyme, cleaves the inactive precursor pro-IL-1β to form the mature active cytokine [36]. When macrophages are exposed to LPS and ATP, they generate ~20–35 times more mature IL-1β than if stimulated with LPS alone [11]. Previous reports have suggested that a $P2X_7$ receptor-induced rise in intracellular Ca^{2+} concentration initiates release of IL-1β-containing microvesicles [12] and/or that $P2X_7$ receptor stimulation by ATP accelerates caspase-1 processing and activation following increased K^+ efflux via a phospholipase A2-dependent mechanism [37], although the exact mechanism of caspase-1 activation is still unclear.

IL-1β has been shown to be responsible for the prominent macrophage infiltration seen in most forms of GN and promotes TNF-α production and glomerular injury in murine GN [38,39]. Interestingly, in the rat model of crescentic GN, maximal $P2X_7$

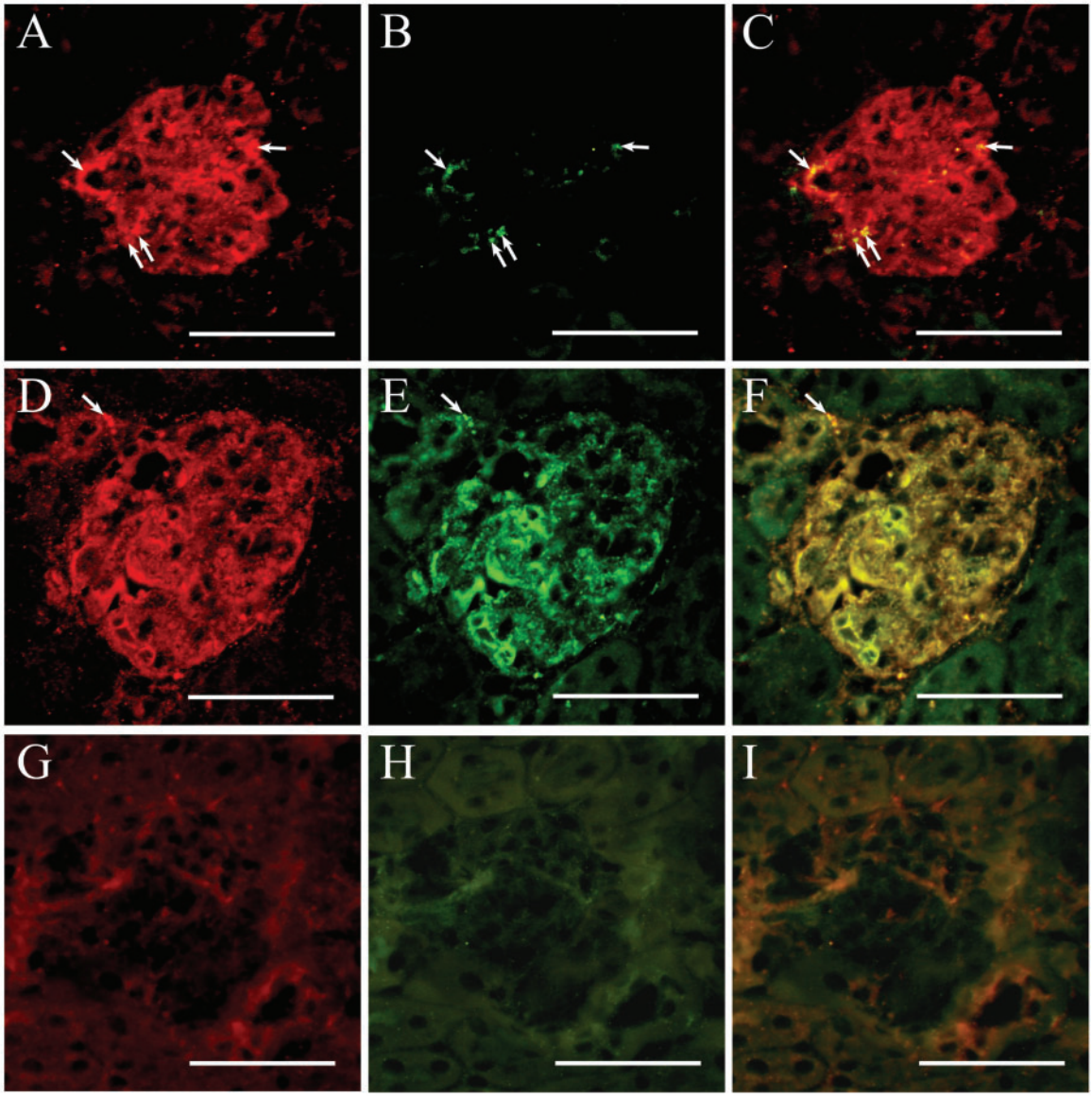

Fig. 4. Double-labelling of P2X$_7$ receptor protein (red) with caspase-3 (green) in mouse accelerated nephrotoxic nephritis. P2X$_7$ receptor immunoreactivity was abundant in glomeruli 24 h post-injection with nephrotoxic serum (**A**), but there was only minimal caspase-3 staining (**B**). The overlay (**C**) does show some double-labelling of P2X$_7$ receptor-positive cells with caspase-3, which appear yellow—the same cells are indicated by arrows in all three plates. Abundant P2X$_7$ receptor (**D**) and abundant caspase-3 positive cells (**E**) are seen in glomeruli and some tubular cells (arrow) 8 days post injection and show double-labelling in the overlay (**F**). (**G**) and (**H**) are sections from a normal mouse glomerulus showing no P2X$_7$ or caspase-3 immunoreactivity, respectively; (**I**) is the overlay (scale bars = 50 μm).

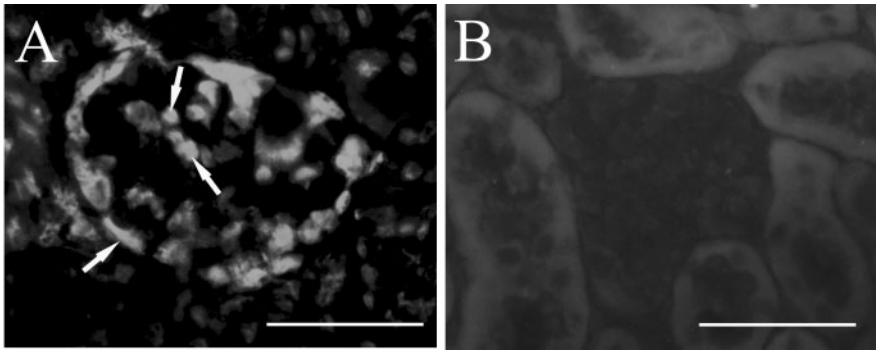

Fig. 5. Increased TUNEL-positive glomerular cells in mouse accelerated nephrotoxic nephritis compared with control. (**A**) TUNEL-positive cells detected in glomeruli and in some surrounding tubule cells of mice with experimental nephrotoxic nephritis (arrows) 8 days post-injection of nephrotoxic serum ($n = 4$), but not in (**B**) normal mouse kidney sections (scale bars = 50 μm).

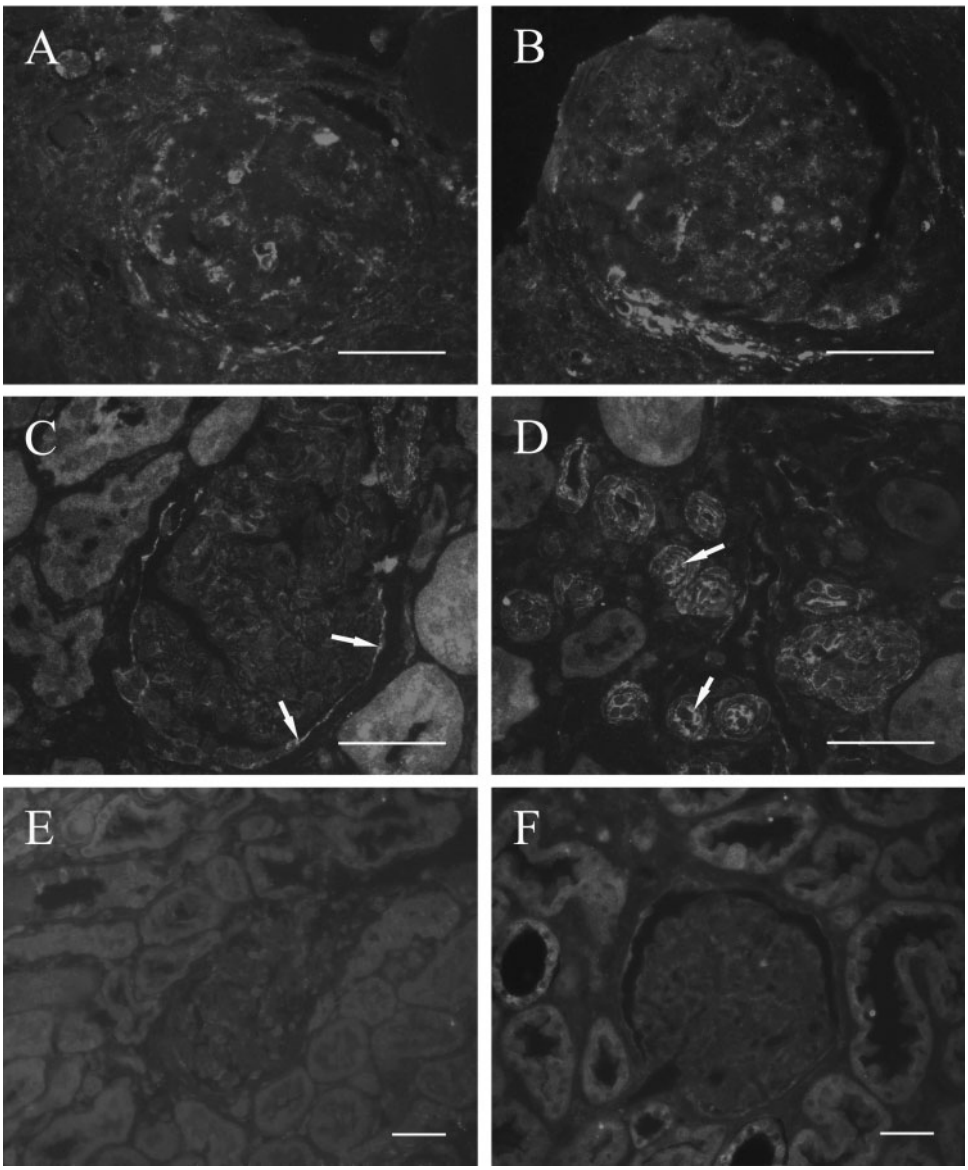

Fig. 6. Expression of $P2X_7$ receptor protein in renal tissues from patients with glomerulonephritis. **(A)** and **(B)** Photomicrographs of renal biopsy tissue from a patient with proliferative lupus glomerulonephritis showing dense and localized $P2X_7$ receptor staining in the glomerulus (scale bars = 50 μm). (**C**) $P2X_7$ receptor staining of Bowman's capsule (arrows) and some dense intracellular proximal tubule staining in a patient with membranous lupus glomerulonephritis (scale bar = 50 μm). (**D**) Photomicrograph of a renal biopsy specimen from a patient with membranous lupus glomerulonephritis, showing mostly apical (arrows) and intracellular $P2X_7$ receptor tubule staining in the outer medulla (scale bar = 50 μm). (**E**) An immunohistochemical control showing no immunostaining with proliferative lupus GN when antibody is pre-absorbed with excess homologous peptide (scale bar = 50 μm). (**F**) Human control kidney showing no $P2X_7$ receptor staining in the tubules or glomeruli (scale bar = 50 μm).

receptor mRNA expression was detected on the 4th day after injection of nephrotoxic serum. We have also shown a significant increase in IL-1β mRNA, which is perhaps not so surprising in an inflammatory model of GN; however, the increase was also maximal by day 4. This coincides with our previous report of glomerular infiltration with monocytes and macrophages, which was also maximal on day 4, as well as the onset of glomerular cell damage and proteinuria in this model [30].

In HEK-293 cells membrane blebbing and micro-vesiculation have been observed within seconds to minutes of $P2X_7$ receptor activation leading to apoptosis [40]. $P2X_7$ receptor-induced processing and release of IL-1β, and its triggering of the apoptotic pathway can occur simultaneously. However, these events can be dissociated, suggesting that they occur via parallel and independent pathways [13]. As an index of apoptosis, we detected caspase-3 positive cells in glomeruli 24 h post-NTS injection, which co-localized with $P2X_7$ immunoreactivity. Furthermore, there was a significant increase in caspase-3 activity 8 days post-NTS, also evident by the significant number of TUNEL-positive cells in

glomeruli 8 days post-NTS injection in the mouse. In addition, there was increased glomerular mRNA expression of the pro-apoptotic markers p53 and bax in rat experimental GN, but no significant difference in the expression of the anti-apoptotic bcl-2 gene. This suggests a link between altered expression of $P2X_7$ receptor and activation of the apoptotic pathway.

ATP is released following cell damage, including renal cell injury, as occurs during the mesangiolytic stage of anti-Thy-1 GN [41]. The length of exposure to the agonist probably determines the response generated by activation of the $P2X_7$ receptor. Activation of the ligand-gated ion channel after brief exposure to ATP may lead to membrane blebbing and apoptosis, and processing and release of IL-1β [20]; whereas, prolonged exposure to ATP leads to formation of a membrane pore which is likely to result in cell death by lysis and necrosis [42]. Whether or not a cell undergoes apoptosis in response to ATP depends on the cell type, the level of $P2X_7$ receptor expression at the cell surface, the concentration of released ATP and the duration of exposure to ATP.

In summary, the pro-inflammatory and pro-apoptotic $P2X_7$ receptor is upregulated in rodent models of GN and in human lupus-related GN. $P2X_7$ receptor mRNA increased on day 4 in a rat model of proliferative GN, which coincided with the onset of proteinuria. We also observed an increase in IL-1β mRNA and in mRNA of the pro-apoptotic markers p53 and bax, as well as an increase in apoptotic cells in glomeruli in the mouse. An increase in $P2X_7$ receptor protein was also detected in glomeruli of the mouse model of accelerated nephrotoxic nephritis, in part due to infiltrating macrophages. In patients with lupus GN, $P2X_7$ receptor protein was detected in glomeruli and renal tubules, but not in control tissue.

In conclusion, our findings suggest that *de novo* renal expression of the $P2X_7$ receptor could be important in the pathogenesis of GN, perhaps through cell loss by apoptosis or the regulation of pro-inflammatory cytokine production. However, selective $P2X_7$ receptor agonists or antagonists suitable for *in vivo* use are not yet available; future work will need to focus on the effect of deleting the $P2X_7$ receptor *in vivo* and *in vitro*.

Acknowledgements. We thank the St Peter's Trust for Kidney, Bladder and Prostate Research (Les Clark Fund) and the Wellcome Trust for support.

Conflict of interest statement. None declared.

References

1. Pusey CD, Peters DK. Immunopathology of glomerular and interstitial disease. In: Schrier RW, Gottschalk CW, eds. *Diseases of the Kidney*, Little, Brown and Co., Boston: 1993; 1647–1680
2. Tam FWK, Pusey CD. The role of T lymphocytes in extracapillary glomerulonephritis. *J Nephrol* 1995; 8: 305–316
3. Tam FWK, Karkar AM, Smith J *et al*. Differential expression of macrophage inflammatory protein-2 and monocyte chemoattractant protein-1 in experimental glomerulonephritis. *Kidney Int* 1996; 49: 715–721
4. Tipping PG, Lowe MG, Holdsworth SR. Glomerular interleukin 1 production is dependent on macrophage infiltration in anti-GBM glomerulonephritis. *Kidney Int* 1991; 39: 103–110
5. Tesch GH, Yang N, Yu H *et al*. Intrinsic renal cells are the major source of interleukin-1 beta synthesis in normal and diseased rat kidney. *Nephrol Dial Transplant* 1997; 12: 1109–1115
6. Matsumoto K, Dowling J, Atkins RC. Production of interleukin 1 in glomerular cell cultures from patients with rapidly progressive crescentic glomerulonephritis. *Am J Nephrol* 1988; 8: 463–470
7. Niemir ZI, Stein H, Dworacki G *et al*. Podocytes are the major source of IL-1 alpha and IL-1 beta in human glomerulonephritides. *Kidney Int* 1997; 52: 393–403
8. Lan HY, Nikolic-Paterson DJ, Zarama M, Vannice JL, Atkins RC. Suppression of experimental crescentic glomerulonephritis by the interleukin-1 receptor antagonist. *Kidney Int* 1993; 43: 479–485
9. Karkar AM, Smith J, Pusey CD. Prevention and treatment of experimental crescentic glomerulonephritis by blocking tumour necrosis factor-alpha. *Nephrol Dial Transplant* 2001; 16: 518–524
10. Grahames CB, Michel AD, Chessell IP, Humphrey PP. Pharmacological characterization of ATP- and LPS-induced IL-1beta release in human monocytes. *Br J Pharmacol* 1999; 127: 1915–1921
11. Le Feuvre RA, Brough D, Iwakura Y, Takeda K, Rothwell NJ. Priming of macrophages with lipopolysaccharide potentiates P2X–mediated cell death via a caspase-1-dependent mechanism, independently of cytokine production. *J Biol Chem* 2002; 277: 3210–3218
12. Gudipaty L, Munetz J, Verhoef PA, Dubyak GR. Essential role for Ca^{2+} in regulation of IL-1beta secretion by $P2X_7$ nucleotide receptor in monocytes, macrophages, and HEK-293 cells. *Am J Physiol Cell Physiol* 2003; 285: C286–C299
13. Verhoef PA, Estacion M, Schilling W, Dubyak GR. $P2X_7$ receptor-dependent blebbing and the activation of Rho-effector kinases, caspases, and IL-1 beta release. *J Immunol* 2003; 170: 5728–5738
14. Rampe D, Wang L, Ringheim GE. $P2X_7$ receptor modulation of beta-amyloid- and LPS-induced cytokine secretion from human macrophages and microglia. *J Neuroimmunol* 2004; 147: 56–61
15. Solle M, Labasi J, Perregaux DG *et al*. Altered cytokine production in mice lacking P2X(7) receptors. *J Biol Chem* 2001; 276: 125–132
16. Labasi JM, Petrushova N, Donovan C *et al*. Absence of the $P2X_7$ receptor alters leukocyte function and attenuates an inflammatory response. *J Immunol* 2002; 168: 6436–6445
17. Hide I, Tanaka M, Inoue A *et al*. Extracellular ATP triggers tumor necrosis factor-alpha release from rat microglia. *J Neurochem* 2000; 75: 965–972
18. Panenka W, Jijon H, Herx LM *et al*. $P2X_7$-like receptor activation in astrocytes increases chemokine monocyte chemoattractant protein-1 expression via mitogen-activated protein kinase. *J Neurosci* 2001; 21: 7135–7142
19. Hillman KA, Burnstock G, Unwin RJ. The P2X(7) ATP receptor in the kidney: a matter of life or death? *Nephron Exp Nephrol* 2005; 101: e24–e30
20. Surprenant A, Rassendren F, Kawashima E, North RA, Buell G. The cytolytic P2Z receptor for extracellular ATP identified as a P2X receptor ($P2X_7$). *Science* 1996; 272: 735–738
21. Rassendren F, Buell G, Virginio C *et al*. The permeabilizing ATP receptor, $P2X_7$. Cloning and expression of a human cDNA. *J Biol Chem* 1997; 272: 5482–5486
22. Di Virgilio F. The P_{2Z} purinoceptor: an intriguing role in immunity, inflammation and cell death. *Immunol Today* 1995; 16: 524–528

23. Coutinho-Silva R, Persechini PM, Bisaggio RD *et al*. P2Z/P2X$_7$ receptor-dependent apoptosis of dendritic cells. *Am J Physiol* 1999; 276: C1139–C1147
24. Harada H, Chan CM, Loesch A, Unwin R, Burnstock G. Induction of proliferation and apoptotic cell death via P2Y and P2X receptors, respectively, in rat glomerular mesangial cells. *Kidney Int* 2000; 57: 949–958
25. Cockcroft S, Gomperts BD. ATP induced nucleotide permeability in rat mast cells. *Nature* 1979; 279: 541–542
26. Virginio C, MacKenzie A, North RA, Surprenant A. Kinetics of cell lysis, dye uptake and permeability changes in cells expressing the rat P2X$_7$ receptor. *J Physiol* 1999; 519: 335–346
27. Collo G, Neidhart S, Kawashima E *et al*. Tissue distribution of the P2X$_7$ receptor. *Neuropharmacology* 1997; 36: 1277–1283
28. Turner CM, Vonend O, Chan C, Burnstock G, Unwin RJ. The pattern of distribution of selected ATP-sensitive P2 receptor subtypes in normal rat kidney: an immunohistological study. *Cells Tissues Organs* 2003; 175: 105–117
29. Vonend O, Turner CM, Chan CM *et al*. Glomerular expression of the ATP-sensitive P2X$_7$ receptor in diabetic and hypertensive rat models. *Kidney Int* 2004; 66: 157–166
30. Tam FWK, Smith J, Morel D *et al*. Development of scarring and renal failure in a rat model of crescentic glomerulonephritis. *Nephrol Dial Transplant* 1999; 14: 1658–1666
31. Tarzi RM, Davies KA, Claassens JW *et al*. Both Fcgamma receptor I and Fcgamma receptor III mediate disease in accelerated nephrotoxic nephritis. *Am J Pathol* 2003; 162: 1677–1683
32. Yang B, Johnson TS, Thomas GL *et al*. Apoptosis and caspase-3 in experimental anti-glomerular basement membrane nephritis. *J Am Soc Nephrol* 2001; 12: 485–495
33. Hillman KA, Woolf AS, Johnson TM *et al*. The P2X$_7$ ATP receptor modulates renal cyst development *in vitro*. *Biochem Biophys Res Commun* 2004; 322: 434–439
34. Ferrari D, Chiozzi P, Falzoni S *et al*. Extracellular ATP triggers IL-1 beta release by activating the purinergic P$_{2Z}$ receptor of human macrophages. *J Immunol* 1997; 159: 1451–1458
35. Perregaux DG, McNiff P, Laliberte R, Conklyn M, Gabel CA. ATP acts as an agonist to promote stimulus-induced secretion of IL-1 beta and IL-18 in human blood. *J Immunol* 2000; 165: 4615–4623
36. Dinarello CA. Interleukin-1 beta, interleukin-18, and the interleukin-1 beta converting enzyme. *Ann NY Acad Sci* 1998; 856: 1–11
37. Kahlenberg JM, Dubyak GR. Mechanisms of caspase-1 activation by P2X$_7$ mediated K^+ release. *Am J Physiol Cell Physiol* 2004; 286: C1100–C1108
38. Timoshanko JR, Kitching AR, Iwakura Y, Holdsworth SR, Tipping PG. Contributions of IL-1beta and IL-1alpha to crescentic glomerulonephritis in mice. *J Am Soc Nephrol* 2004; 15: 910–918
39. Timoshanko JR, Kitching AR, Iwakura Y, Holdsworth SR, Tipping PG. Leukocyte-derived interleukin-1beta interacts with renal interleukin-1 receptor I to promote renal tumor necrosis factor and glomerular injury in murine crescentic glomerulonephritis. *Am J Pathol* 2004; 164: 1967–1977
40. Wilson HL, Wilson SA, Surprenant A, North RA. Epithelial membrane proteins induce membrane blebbing and interact with the P2X$_7$ receptor C terminus. *J Biol Chem* 2002; 277: 34017–34023
41. Poelstra K, Heynen ER, Baller JF, Hardonk MJ, Bakker WW. Modulation of anti-Thy1 nephritis in the rat by adenine nucleotides. Evidence for an anti-inflammatory role for nucleotidases. *Lab Invest* 1992; 66: 555–563
42. Buisman HP, Steinberg TH, Fischbarg J *et al*. Extracellular ATP induces a large nonselective conductance in macrophage plasma membranes. *Proc Natl Acad Sci USA* 1988; 85: 7988–7992

Received for publication: 27.1.06
Accepted in revised form: 7.9.06