P2X<sub>7</sub> Deficiency Attenuates Renal Injury in Experimental Glomerulonephritis


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ABSTRACT
The P2X<sub>7</sub> receptor is a ligand-gated cation channel that is normally expressed by a variety of immune cells, including macrophages and lymphocytes. Because it leads to membrane blebbing, release of IL-1β, and cell death by apoptosis or necrosis, it is a potential therapeutic target for a variety of inflammatory diseases. Although the P2X<sub>7</sub> receptor is usually not detectable in normal renal tissue, we previously reported increased expression of both mRNA and protein in mesangial cells and macrophages infiltrating the glomeruli in animal models of antibody-mediated glomerulonephritis. In this study, we used P2X<sub>7</sub>-knockout mice in the same experimental model of glomerulonephritis and found that P2X<sub>7</sub> deficiency was significantly renoprotective compared with wild-type controls, evidenced by better renal function, a striking reduction in proteinuria, and decreased histologic glomerular injury. In addition, the selective P2X<sub>7</sub> antagonist A-438079 prevented the development of antibody-mediated glomerulonephritis in rats. These results support a proinflammatory role for P2X<sub>7</sub> in immune-mediated renal injury and suggest that the P2X<sub>7</sub> receptor is a potential therapeutic target.


Glomerulonephritis (GN) is a major cause of end-stage kidney disease; current therapy usually involves relatively nonspecific immunosuppression with often serious adverse effects. Glomerular deposition of antibodies directed against exogenous antigens or autoantigens, leading to immune complex–mediated inflammation and tissue injury, has been well documented in both experimental and clinical forms of GN.

The rat model of nephrotoxic nephritis (NTN) has demonstrated the importance of IL-1β in GN; renal levels of IL-1β are increased in this form of GN, and IL-1β has been shown to play an important role in glomerular crescent formation and in subsequent tubulointerstitial injury. Moreover, early and late treatment with an IL-1 receptor antagonist prevents the progression of crescentic GN, or IL-18<sup>−/−</sup> mice, and treatment with caspase inhibitors reduces renal inflammation and apoptosis—all consistent with a central role for IL-1β in this experimental model of GN.

The ATP-sensitive P2X<sub>7</sub> receptor is a cation channel activated by high concentrations of extracellular ATP. Stimulation of this receptor is proinflammatory and can lead to tissue damage and organ failure. In this study, we investigated the role of P2X<sub>7</sub> in experimental glomerulonephritis and found that P2X<sub>7</sub> deficiency provided significant renoprotection compared with wild-type controls.
flamatory, causing release of inflammatory cytokines such as IL-1β and IL-18 from macrophages, changes in plasma membrane lipid distribution, and cell death by necrosis or apoptosis.10,11 A central role for P2X7 in IL-1β secretion via the Nalp Domain-, Leucine-Rich Repeat-, and PYD-Containing Protein 3 (NALP3) inflammasome has been shown in P2X7-deficient mice.12,13 This receptor also has significant prothrombotic effects,14 causing release of tissue factor-bearing microparticles.15 Indeed, P2X7 is already considered to be a possible therapeutic target in inflammation, and antagonists are currently in Phase II clinical trials for the treatment of rheumatoid arthritis and chronic obstructive pulmonary disease; however, the role of this receptor in renal disease or injury is still unclear.16

We previously reported an increase in glomerular expression of the P2X7 receptor (at the mRNA and protein levels) in rats and mice with NTN induced by nephrotoxic globulin (NTG)—an established model of immune complex–mediated GN characterized by proteinuria, glomerular thrombosis, and tubulointerstitial injury—as well as in renal biopsy tissue from patients with lupus nephritis.17,18 In this study, we used P2X7-deficient mice and the selective P2X7 antagonist A-438079 to examine in more detail the role of P2X7 in the NTN model of GN.

RESULTS

Mice lacking P2X7 develop normally, are of similar weight to wild-type littermates used as controls, and have normal macroscopic and microscopic renal morphology and histology. At day 9 after injection of NTG, glomerular thrombosis (as indicated by periodic acid-Schiff [PAS]-positive fibrin)19 was reduced in P2X7−/− mice compared with controls (Figure 1, A through D). Quantification of glomerular thrombosis revealed a 60% reduction in the P2X7−/− mice compared with controls (P < 0.01; Figure 1E). Consistent with less severe histologic injury, P2X7−/− mice at day 8 had a 52% reduction of proteinuria (P < 0.05; Figure 1F) as well as a 38% reduction in serum creatinine levels measured after terminal bleeding on day 9 (P < 0.05; Figure 1G).

The Accelerated Nephrotic Nephritis (ANTN) model depends on both the immune response to the injected sheep IgG before administration of the NTG and deposition of the injected NTG within the kidney. To assess the former, we quantified circulating levels of mouse anti-sheep IgG, whereas, for the latter, we quantified glomerular deposition of sheep IgG in each experimental group (Supplemental Figure 1). There were no differences in sheep IgG deposition between P2X7−/− and controls (Supplemental Figure 1E), and circulating levels of mouse anti-sheep IgG were also similar (Supplemental Figure 1G).

Although the immune response to sheep IgG did not differ between the two experimental groups of mice, glomerular deposition of mouse IgG was reduced by 26% in P2X7−/− mice compared with controls (P < 0.05; Supplemental Figure 1F); however, for every measure of disease activity, mice had significantly higher levels of activity for the same level of mouse anti-sheep IgG deposition, suggesting that this is not the explanation for the reduced injury in the P2X7−/− mice (see Supplemental Figure 2).

Macrophages are the major effector cells in human and experimental GN.20 P2X7 has a central role in macrophage IL-1β secretion via the NALP3 inflammasome.19 Monocyte chemoattractant protein 1 (MCP-1) is expressed by macrophages, endothelial cells, mesangial cells, and epithelial cells,21 and this

Figure 1. Glomerular thrombosis, proteinuria, and serum creatinine levels in wild-type and P2X7−/− mice. (A through D) Representative low- and high-power microscopy of PAS-stained sections of kidneys from wild-type (A and B) and P2X7−/− (C and D) mice 9 d after administration of nephrotoxic serum. (E) P2X7−/− mouse kidneys showed significantly reduced glomerular thrombosis compared with the wild-type, confirmed by quantification of thrombosis scores. (F and G) Proteinuria was also significantly reduced in P2X7−/− mice killed 9d after administration of nephrotoxic serum compared with controls (F), as were serum creatinine levels (G).
chemokine has been shown to be important in glomerular recruitment of macrophages and crescent formation in NTN in WKY rats, as well as in mice. We measured cytokine and chemokine levels in the urine using specific ELISAs. IL-1β levels proved too low for accurate detection (data not shown), but MCP-1 levels were readily detectable and were reduced by 96% in P2X$_7^{-/-}$ mice compared with controls ($P < 0.0001$; Figure 2A). To determine whether decreased MCP-1 levels in P2X$_7^{-/-}$ mice were associated with reduced macrophage recruitment, we quantified glomerular macrophage infiltration (Figure 2B). As expected, this demonstrated a significant reduction in macrophage numbers per glomerular cross-section in P2X$_7^{-/-}$ mice compared with controls (28%; $P < 0.001$; Figure 2C). In contrast, interstitial macrophages were not reduced in the P2X$_7^{-/-}$ mice compared with controls (9.29 ± 1.13 versus 8.57 ± 1.05 macrophages per high-power view).

We next investigated complement activation by measuring C3 deposition to determine whether this might explain reduced MCP-1 production in P2X$_7^{-/-}$ mice. We found no significant differences between P2X$_7^{-/-}$ and control mice (Figure 2E and Supplemental Figure 3, A and B), suggesting that differential activation of complement is not responsible for the reduction in MCP-1; however, fibrin deposition was reduced by 24% in P2X$_7^{-/-}$ mice compared with control mice ($P < 0.05$; Figure 2D and Supplemental Figure 3, C and D).

A-438079 is a selective P2X$_7$ antagonist in rat tissue in vitro and in vivo models of pathologic nociception. To assess the potential of P2X$_7$ as a therapeutic target in vivo, we used this antagonist in the rat NTN model to determine whether pharmacologic inhibition of the P2X$_7$ receptor can affect the course of NTN. Two dosages were used: a “low dosage” of 100 μmol/kg and a “high dosage” of 300 μmol/kg, based on previous reports. At day 7 after injection of nephrotoxic serum (NTS), glomerular thrombosis (as indicated by PAS-positive fibrin) was not significantly reduced in rats treated with low-dosage A-438079 but was reduced by 96% in rats treated with high-dosage A-438079 compared with vehicle-injected controls ($P < 0.01$; Figure 3A). Rats treated with high-dosage A-438079 also had a 90% reduction in proteinuria at days 6 to 7 compared with controls ($P < 0.001$; Figure 3B).

We then assessed whether P2X$_7$ inhibition affected the immune response to NTS administration. There were no significant differences in rabbit IgG and rat IgG deposition between rats treated with either dosage of A-438079 and controls (Supplemental Figure 4). As expected from our results in P2X$_7$-deficient mice, macrophage infiltration of glomeruli was reduced in animals treated with high-dosage A-438079, the number of macrophages per glomerulus being reduced by 65% ($P < 0.001$; Figure 4). Similarly, renal levels of MCP-1 were reduced by 50% in animals treated with high-dosage A-438079 ($P < 0.05$; Supplemental Figure 5).

These results clearly demonstrate that P2X$_7$ deficiency protects against the inflammatory damage that occurs in ANTN; P2X$_7$-deficient mice develop less severe glomerular thrombosis and proteinuria and have lower levels of serum creatinine when compared with their controls. Similarly, the selective P2X$_7$ antagonist A-438079 pre-
vents the development of NTN in WKY rats in a dosage-dependent manner. Both P2X<sub>7</sub>-deficient mice and rats treated with A-438079 have reduced macrophage infiltration of renal tissue and express markedly lower renal or urinary levels of the chemokine MCP-1. Interestingly, in a model of unilateral ureteric obstruction in another line of P2X<sub>7</sub> gene–deficient mice, Goncalves et al. also observed reduced renal macrophage infiltration, as well as decreased TGF-β1 synthesis and collagen deposition.

**DISCUSSION**

Macrophages are important effectors of injury in GN. Accumulation of macrophages is linked closely to the severity of glomerular injury, and macrophages are a major source of IL-1β and IL-18, both known to play an important role in crescent formation and tubulointerstitial injury. MCP-1 is a key molecule responsible for recruitment of macrophages to inflamed glomeruli; it is expressed by both macrophages and intrinsic renal cells.

In line with our finding of decreased urinary MCP-1 in P2X<sub>7</sub>−/− mice in ANTN and reduced renal MCP-1 in WKY rats treated with the selective P2X<sub>7</sub> antagonist A-438079, others have reported reduced tissue MCP-1 production in an adjuvant-induced model of paw inflammation in P2X<sub>7</sub>−/− mice. Furthermore, the P2X<sub>7</sub> agonist BzATP [3′-O-(4-benzoyl)benzoyl ATP] increases MCP-1 expression in macrophage-like astrocytes; therefore, reduced MCP-1 production—and urinary excretion—could be a consequence of P2X<sub>7</sub> deficiency itself or secondary to a decrease in IL-1β secretion.

In conclusion, our findings indicate a significant and possibly key proinflammatory role for the P2X<sub>7</sub> receptor in autoimmune renal injury and provide evidence that P2X<sub>7</sub> antagonists can prevent the development of disease, confirming that P2X<sub>7</sub> is an important novel therapeutic target in GN.

**CONCISE METHODS**

**Animals**

P2X<sub>7</sub>-deficient mice were a gift from GlaxoSmithKline and have been described in detail elsewhere. Mice were kept in a pathogen-free environment, and experiments were performed according to local institutional guidelines. All mice were female and of 6 to 8 wk of age. Mice were initially bred from P2X<sub>7</sub>−/− breeding pairs; we studied P2X<sub>7</sub>−/− mice and used P2X<sub>7</sub>+/+ littermates as controls. Male WKY rats that weighed between 180 and 220 g were used. Animals had free access to standard laboratory diet and water.

**Experimental Design**

To induce ANTN, we immunized mice with a single intraperitoneal injection of 200 μg of sheep IgG (Sigma, Gillingham, UK) in a 50:50 mix with complete Freund’s adjuvant (Sigma, Poole, UK). This was followed 5 d later by the intravenous administration of 200 μl of sheep NTG via the tail vein. Preparation of NTG was performed as described previously. NTN was induced in WKY rats by single intravenous injection of 0.1 ml of rabbit anti-rat NTS. In P2X<sub>7</sub> antagonist experiments, the first dose of A-438079 was administered on the same day (at a dosage of either 100 or 300 μmol/kg), and further doses were given twice daily for an additional 7 d. Mice were killed at 9 d and rats at 7 d after the administration of NTG or NTS, respectively.

**Reagents**

A-438079 has a molecular weight of 342.61; the chemical name is 3-[[5-(2,3-dichlorophenyl)-1H-tetrazol-1-yl]methyl]pyridine hydrochloride (pIC<sub>50</sub> = 6.5 for recombinant rat P2X<sub>7</sub>). Two dosages were chosen, 100 and 300 μmol/kg, based on previously reported data.

**Measurement of Disease**

Mice and rats were housed in metabolic cages for 24-h collection of urine. The protein concentration was measured using the sulfoal-

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**Figure 3.** Effect of early treatment with the P2X<sub>7</sub> antagonist A-438079 on NTN in rats. (A) Rats treated with the 300-μmol/kg dose of A-438079 had a significant reduction in fibrinoid necrosis compared with vehicle-treated rats, whereas rats treated with the 100-μmol/kg dose showed only a trend toward lower levels of disease. (B) Similarly, rats treated with the higher dosage of A-438079 had a significant reduction in proteinuria compared with the vehicle-treated group, whereas rats treated with the lower dosage did not have significantly lower levels.
The cyclic acid method, in which 10 µl of 25% sulfosalicylic acid was added to 100 µl of urine diluted to 1:100, and absorbance was read at 450 nm. Serum creatinine was measured using an Olympus AU600 analyzer (Olympus, Watford, Hertfordshire, UK). Samples of kidney were either unfixed or fixed in periodate-lysine-paraformaldehyde and snap-frozen in isopentane precooled in liquid nitrogen or fixed in Bouin’s solution and then embedded in paraffin for sectioning.

**IL-1β and MCP-1 Estimation**

DuoSet ELISA kits (R&D Systems, Minneapolis, MN) were used according to the manufacturer’s instructions. The antibody supplied in the IL-1β kit does not cross-react with pro–IL-1β. Sandwich ELISA was performed to assess the presence of rat MCP-1 in renal tissue homogenate. Briefly, matched paired antibodies from BD Pharmingen (San Diego, CA) were used according to the manufacturer’s instructions. Absorbance was read at 450 nm using a spectrophotometric ELISA plate reader (Anthos HTII; Anthos Labtec, Salzburg, Austria).

**Histologic Analysis**

Kidneys were fixed for 2 h in Bouin solution, transferred to 70% ethanol, and embedded in paraffin. Sections were then stained with PAS. All analyses were performed blind. Glomerular thrombosis was assessed by grading the degree of PAS-positive material per glomerulus as follows: Grade 0, no PAS-positive material within the glomerulus; grade 1, 0 to 25% of glomerulus thrombosed; grade 2, 25 to 50%; grade 3, 50 to 75%; and grade 4, 75 to 100%. The mean thrombosis score for 50 glomeruli was then calculated.

For detection of macrophages, slides were dewaxed and rehydrated, boiled with 0.01 M sodium citrate buffer for 15 min, and then immersed in 50% methanol containing 0.3% H2O2 to block endogenous peroxidase activity. Slides were washed with PBS and then incubated with 20% normal goat serum. The primary antibody was mAb ED-1 (Serotec, Kidlington, Oxfordshire, UK) diluted 1:500 and incubated overnight. Slides were then incubated with biotinylated goat anti-mouse (Dako, Ely, Cambridgeshire, UK) for 45 min, followed by avidin-peroxidase conjugate (Vector Labs, Orton Southgate, Peterborough, UK) for 30 min. Antibody binding was visualized using 3,3′ diaminobenzidene (Sigma Aldrich, Gillingham, Dorset, UK) and counterstained with hematoxylin. Macrophages were counted in 25 glomerular cross-sections per sample, and the mean number of macrophages was calculated.

For detection of rat MCP-1, staining slides were dewaxed and rehydrated, boiled with 0.01 M sodium citrate buffer for 15 min, and then immersed in 0.3% H2O2 to block endogenous peroxidase activity. Slides were then washed with PBS and then incubated with 20% normal swine serum. The MCP-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was diluted 1:50 with 5% rat serum and incubated overnight. Slides were then incubated with biotinylated swine anti-goat antibody (Dako, Ely, Cambridgeshire, UK), diluted in 5% rat serum for 1 h, followed by extravidin peroxidase (Sigma Aldrich) for 30 min. Antibody binding was visualized using 3,3′ diaminobenzidene (Sigma Aldrich).

**Figure 4.** Effect of early treatment with the P2X7 antagonist A-438079 on infiltrating macrophages. Macrophage infiltration was assessed by immunoperoxidase staining using ED-1 mAb. (A) Glomeruli from vehicle-treated rats and those on the lower dosage of 100 µmol/kg A-438079 showed intense macrophage infiltration, whereas macrophage infiltration was significantly reduced in rats given the higher dosage of 300 µmol/kg of the P2X7 antagonist A-438079. (B) Macrophage infiltration as assessed by counting the number of infiltrating macrophages per GCS in 25 consecutive glomeruli confirmed these results.
Direct Immunofluorescence Microscopy
Detection of glomerular mouse and sheep IgG in mouse tissue and rat and rabbit IgG in rat tissue was assessed on frozen sections by direct immunofluorescence microscopy. Briefly, sections were fixed in acetone for 10 min, blocked with 10% normal goat serum, and then incubated with FITC-conjugated anti-IgG antibodies (Sigma Aldrich). FITC-labeled goat anti-mouse C3 (cat. no. 55500; Cappel/ICN, Aurora, OH) and FITC-labeled goat anti-mouse Fibrinogen (Nordic Immunology, Tilburg, The Netherlands) were used to detect C3 and fibrin deposition, respectively. For quantification of glomerular immunofluorescence, sections were examined using an Olympus BX4 fluorescence microscope (Olympus Optical, London, UK) at ×20 magnification. Images were captured using a Photonic Science Color Coolview camera (Photonic Sciences, Robertsbridge, UK) and analyzed using Image Pro Plus software (Media Cybernetics, Silver Spring, MD). For each section, 20 consecutive glomeruli were analyzed and the mean fluorescence intensity was calculated.

Homogenization of Frozen Kidney Tissues
Snap-frozen renal tissue was homogenized and sonicated with homogenizing buffer (20 mM Tris-HCL [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton-X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, and 1 mM sodium orthovanadate plus 1% protease inhibitor cocktail; Sigma Aldrich) at a concentration of 0.1 g of tissue with 1 ml of buffer. The homogenate was then centrifuged to remove tissue debris. Protein concentration was determined using the bicinchoninic acid assay kit (Pierce, Rockford, IL) plus 1% protease inhibitor cocktail; Sigma Aldrich). For each section, 20 consecutive glomeruli were analyzed and the mean fluorescence intensity was calculated.

Statistical Analysis
All values described in the text and figures are expressed as means ± SEM. Statistical analysis was carried out by GraphPad Prism 5.01 (GraphPad Software, San Diego, CA). Data were analyzed by the Mann-Whitney U test; P < 0.05 was considered to be significant.

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