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Glomerular expression of the ATP-sensitive $P2X_7$ receptor in diabetic and hypertensive rat models

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Background. The molecular identification and characterization of the adenosine triphosphate (ATP)-sensitive family of P2 receptors is comparatively new. There are two main subgroups, each with several subtypes and widespread tissue distribution, including the kidney. A unique member of the P2X subgroup of P2 receptors is the ATP-gated ion channel $P2X_7$, which on activation can cause cell blebbing, cytokine release, and cell death by necrosis or apoptosis. We report expression of this receptor in normal rat kidney and in two chronic models of glomerular injury: streptozotocin-induced (STZ) diabetes and ren-2 transgenic (TGR) hypertension.

Methods. At different time points in these models, we used a polyclonal antibody to the $P2X_7$ receptor and immunohistochemistry to determine its expression and distribution. We also used Western blotting and real-time polymerase chain reaction (PCR) to detect changes in $P2X_7$ receptor protein and mRNA expression, respectively.

Results. We found only low-level glomerular immunostaining for the $P2X_7$ receptor in normal rat kidney, but intense $P2X_7$ receptor immunostaining of glomeruli in kidneys from diabetic animals at 6 and 9 weeks, and in hypertensive animals at 12 weeks. In diabetic animals, real-time PCR demonstrated a ~tenfold increase in glomerular $P2X_7$ receptor mRNA relative to control, and Western blotting confirmed an increase in protein. Immunohistochemistry and immunoelectron microscopy showed staining of glomerular podocytes, which was both intracellular and at the plasma membrane.

Conclusion. We conclude that the $P2X_7$ receptor is not expressed appreciably under normal conditions, but that following glomerular injury it is significantly up-regulated, mainly in podocytes, though also in endothelial and mesangial cells, of animals with STZ-induced diabetes mellitus or TGR hypertension. Although the exact function and regulation of this receptor remain unclear, its association with inflammatory cytokine release and cell death suggests that increased expression might be involved in the pathogenesis of glomerular cell injury or repair.

Extracellular adenosine triphosphate (ATP) and adenosine were first recognized to have important biologic actions over 70 years ago, and the concept that these purines may act as paracrine or autocrine mediators and regulators of cellular function was put forward more than 40 years later [1]. In 1978, Burnstock proposed an early classification of purine receptors, distinguishing between those for adenosine (P1) and ATP (P2) [2]. The P2 receptors have since been divided into two major subgroups, P2X and P2Y, which are based on their molecular structures, intracellular signal transduction mechanisms, and pharmacological profiles [3]. Currently, seven members of the P2X family have been identified and eight subtypes of the P2Y receptor [4, 5].

The $P2X_{1-7}$ receptors share a sequence homology of around 35% to 50%, but the $P2X_7$ receptor is more structurally divergent and unique, having a much longer C-terminus (239 amino acids) than the other six P2X receptors [6]. The $P2X_7$ receptor, believed to be the original P2Z receptor [7], has a dual response to extracellular ATP: rapid opening of a ligand-gated cation channel (a typical P2X response), followed by induction of a large cytoplasmic pore permeable to molecules of between 600 and 900 Da [6]. It is known that extracellular ATP can kill cells by either necrosis and/or apoptosis and that this is probably mediated via the $P2X_7$ receptor [5]. Moreover, at least in lymphoid cells, low-level expression of the $P2X_7$ receptor has been linked to cell proliferation [8], as well as cell death, and (though still controversial) that receptor polymorphisms may affect prognosis in chronic lymphocytic leukemia [9] and in tuberculosis [10]. However,

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the physiologic and pathologic role of this receptor, its regulation and tissue expression, remain unclear.

In the present study, we have examined the distribution and localization of the $P2X_7$ receptor in normal kidneys and in kidneys from two rat models of glomerular injury due to diabetes or hypertension, respectively, using immunohistochemistry with a rabbit polyclonal antibody to the rat $P2X_7$ receptor, as well as real-time polymerase chain reaction (PCR) detection of its mRNA (in the diabetic model only). We chose these models because of previous findings that mesangial cells [11, 12] and glomerular epithelial cells [13] can express the $P2X_7$ receptor and that high glucose altered its expression in cultured fibroblasts [14].

METHODS

Rat models

Model of diabetes. Dr. E. Debnam (University College London) provided the streptozotocin (STZ)-induced diabetic rat kidneys. Male Sprague-Dawley rats (initial weight 240 g, aged 6 to 7 weeks) were given a single injection of STZ into a tail vein (50 to 60 mg/kg dissolved in pH 4.5 citrate buffer) under light anesthesia [15]. The animals were then sacrificed at 3, 6, and 9 weeks post-injection and their kidneys examined by immunocytochemistry; isolated glomeruli were obtained for protein and mRNA extraction. Kidneys from age-matched control rats were also examined. In STZ-induced diabetic rats, glomerular filtration rate (GFR) and renal blood flow at 8 weeks post-STZ are increased compared with control [16]; glomerular morphology at 14 weeks post-STZ is no different from control and the majority of glomeruli look normal under the light microscope [17]. Although we did not record blood pressure in our treated rats, an earlier and representative study of this model, using a similar dose of STZ, found no change in diastolic or mean blood pressure compared with a control group for up to 10 weeks [18].

Model of hypertension. The kidneys of hypertensive transgenic (mRen2) 27 rats (TGR), which expresses the murine *Ren-2* gene [19], were kindly provided by Professor J. Mullins (Edinburgh University) and Dr. J. McEwan (University College London), and additional archived renal tissue by Professor S. Fleming (University of Dundee). At the time of sacrifice these animals were aged 12 weeks. This animal model is no longer accessible for further study and the kidney tissue available to us was examined by immunocytochemistry only. TGR rats develop severe hypertension, which begins at 4 weeks and peaks at 9 weeks of age [19]; blood pressure decreasing thereafter [20]. No renal damage is visible up to 10 weeks of age [21]; although GFR is no different from control at 16 and 32 weeks, glomerulosclerosis is present, and by 18 weeks >50% of glomeruli are sclerotic and the intrarenal vessels thickened [20, 21].

Immunohistochemistry

Kidney tissue was embedded in Tissue-Tek (Sakura Finetek, The Netherlands) and frozen in isopentane precooled in liquid nitrogen. The unfixed cryostat sections (8 μm thick) were collected on poly-L-lysine-coated slides and allowed to equilibrate at room temperature for at least 10 minutes and then fixed in 4% formaldehyde-0.03% picric acid in 0.1 mol/L phosphate buffer (pH 7.4) for 2 minutes. From archived tissue, sections (4 μm) were cut, placed on gelatin-coated slides, and dried overnight at 37°C; sections were then dewaxed with xylene and rehydrated in decreasing concentrations of ethanol.

After blocking the endogenous peroxidase activity in 50% methanol-0.3% hydrogen peroxide (H_2O_2) for 15 minutes, specimens were incubated overnight at room temperature with the primary $P2X_7$ receptor antibody [5 μg/mL in 10% normal horse serum (NHS)/phosphate-buffered saline (PBS)], as described previously [22]. The secondary antibody was a biotinylated donkey antirabbit IgG (Jackson ImmunoResearch, Luton, UK) ExtrAvidin-horseradish peroxidase (Sigma, Poole, UK) was used to increase the sensitivity of antigen localization. Nickel-intensified diaminobenzidine tetrahydrochloride (DAB) and H_2O_2 were used as the enzyme substrate to produce a black amorphous reaction product in the sections. Control experiments were performed to establish specific immunoreactivity: sections were incubated with $P2X_7$ receptor antibody pretreated with an excess of the homologous peptide antigen; the primary antibody was replaced with nonimmune rabbit antiserum, or without primary antibody. The $P2X_7$ receptor antibody was obtained from Roche Bioscience (Palo Alto, CA, USA). The subtype-selective antibody was raised in rabbits against a specific 15 amino acid residue at the carboxy-terminus of the P2X receptor molecule [23]. We confirmed in human embryonic kidney (HEK) cells expressing recombinant rat $P2X_7$ that this antibody detects a ~80 kD band (not shown), which is thought to be the glycosylated monomeric form of the receptor [24]; in rat brain this same band was detected plus a ~50 kD, also reported previously [25], which (tested because of its predicted size) when detected in rat kidney tissue (see **Results** section) did not cross-react with a Roche $P2X_4$ polyclonal antibody (not shown); we also confirmed no glomerular immunostaining with this antibody in normal [26] and diabetic (not shown) kidney. Anti-thymocyte-1 (Thy-1) (Abcam, Cambridge, UK) antibody was used as a marker for mesangial cells and anti-Wilms tumor-1 (WT-1) (Santa Cruz Biotechnlogy, Santa Cruz, CA, USA) antibody was used as a marker for podocytes. The secondary antibody for fluorescence microscopy was either the fluorescein isothiocyanate (FITC)-conjugated antibody (ICN, Biomed, CA, USA) (green color) or a Cy3-labeled anti-rabbit IgG (Abcam) (red color). Antibodies to the $P2X_7$ receptor and the podocyte marker WT-1 were

raised in the same species, and additional steps were required to ensure elimination of cross-reactivity. After incubation overnight with anti-$P2X_7$ receptor antibody, the layers of secondary antibody were biotinylated donkey anti-rabbit IgG (Jackson Immunoresearch) and ExtrAvidin peroxidase (Sigma); the tyramide signal amplification solution was applied for 8 min and the final layer was streptavidin fluorescein (Amersham Lifescience, Bucks, UK). Finally, sections were incubated overnight with anti-WT-1 and then detected with donkey antirabbit Cy3. Pictures were taken with a Zeiss Axioplan immunofluorescent microscope with a Leica DC200 digital camera (Zeiss, Oberkochen, Germany).

Immunoelectron microscopy

Blocks (about 0.5 × 0.5 cm) of kidney were dissected out and immersion-fixed overnight at 4°C in fixative consisting of 4% paraformaldehyde and 0.2% glutaraldehyde in 0.1 mol/L phosphate buffer (pH 7.4). The following day, the specimens were rinsed in phosphate buffer for several hours (at 4°C) and then transferred to 0.05 mol/L Tris-buffered saline (TBS) at pH 7.6. Sections (60 to 70 μm) were cut on a vibratome, collected in TBS, and processed for the pre-embedding electron immunocytochemistry of $P2X_7$ receptor antibody, using the ExtrAvidin peroxidase-conjugate procedure as previously described [27]. Initially, sections were washed in TBS and exposed for 30 minutes to 0.3% H_2O_2 in 30% ethanol to block endogenous peroxidases and then washed in TBS. The main steps of the immunoprocedure included (*1*) for blocking nonspecific protein binding sites, incubation of sections for $1^1/_2$ hours with heat-treated 10% NHS (Jackson ImmunoResearch); (*2*) incubation for 20 hours with a rabbit polyclonal antibody to the $P2X_7$ receptor (at 3 μg antibody/mL of TBS containing 10% NHS and 0.1% sodium azide); (*3*) incubation for 5 hours with a biotin-conjugated donkey-antirabbit immunoglobulin G (IgG) (H + L) serum (Jackson ImmunoResearch) diluted 1:500 in TBS containing 1% NHS and 0.1% sodium azide; and (*4*) incubation for 18 hours with ExtrAvidin-horseradish peroxidase conjugate (Sigma-Aldrich, Poole, UK) diluted 1:1500 in TBS. After exposure to DAB and H_2O_2, and osmication (1% osmium tetroxide in 0.1 mol/L cacodylate buffer, pH 7.4), the specimens were dehydrated in a graded series of ethanol and embedded in Araldite. The ultrathin sections were stained with uranyl acetate and lead citrate and examined with a JEM-1010 electron microscope.

Controls for electron microscopy-immunocytochemistry

No immunolabeling was observed when the $P2X_7$ receptor antibody was omitted from the incubation medium and/or replaced with nonimmune NHS and nonimmune normal rabbit serum (Nordic Immunology, Tilberg, The Netherlands), or when the biotin-conjugated donkey-antirabbit IgG serum was omitted from the incubation medium.

Identification of apoptosis in glomeruli by the TUNEL assay

The terminal deoxynucleotidyl transferase-mediated nick end-labeling (TUNEL) assay was performed using the In Situ Cell Death Detection Kit (Roche Molecular Biochemicals, Mannheim, Germany), according to the manufacturer's instructions. Briefly, sections were fixed with 4% formaldehyde for 20 minutes and then washed three times with PBS. Cells were permeabilized with 0.1% Triton X-100, 0.1% sodium citrate solution in PBS for 2 minutes at 4°C. Sections were rinsed three times with PBS and then incubated for 1 hour in TUNEL reaction mixture. After a further washing in PBS, the slides were then mounted in Citifluor and examined with a Zeiss Axioplan immunofluorescent microscope.

Glomerular mRNA and protein extraction

Glomeruli were isolated from the renal cortex by a serial sieving technique. Pieces of cortex were minced, pushed through a series of stainless steel mesh sieves of different pore sizes (150, 106, and finally 75 μm) and rinsed with ice-cold Hepes buffer; collected, divided and centrifuged at 1500 × *g* for 8 minutes. Sieved tissue was >90% free of extraglomerular components.

For protein extraction, lysis buffer containing 50 mmol/L Tris, pH 8, 75 mmol/L NaCl, 1% Nonidet, 10 mmol/L deoxycholate, 0.1% sodium docedyl sulfate (SDS), 25 μg mL^{-1} leupeptin, 200 units mL^{-1} aprotinin, 1 μmol/L pepstain A, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF) (Sigma) was added and the mixture incubated in ice for 1 hour. The tubes were centrifuged at 14000 × *g* for 15 minutes and the supernatant was used for Western blotting. The protein concentration was estimated using a standard BCA method (Pierce, Rockford, IL, USA).

Total RNA was extracted with TRIzol (Invitrogen, Renfrewshire, UK). The amount of RNA was estimated using a spectrophotometer set at 260/280 nm. Messenger RNA was isolated using oligo-dT–coated magnetic beads (PolyATract) (Promega, Madison, WI, USA) according to the manufacturer's protocol. Synthesis of cDNA was done with an equivalent of 2 μg RNA, oligo-dT primer and 1 U Superscript II RNase H^- reverse transcriptase (Invitrogen), according to the supplier's protocol.

Detection and quantification of $P2X_7$ receptor and β-actin using real-time PCR

The resulting cDNA transcripts of glomerular mRNA were used for PCR amplification by PCR. To ensure primer specificity, PCR products were also analyzed by

gel electrophoresis in the BioRad Multi-Imager (BioRad, Hercules, CA, USA). In these semiquantitative experiments, the PCR was performed for 35 or 20 cycles for the $P2X_7$ receptor and β-actin, respectively. For real-time PCR quantification, we used the BioRad Icycler (BioRad) with a PCR-mix containing SYBR-Green I. SYBR-Green I is a useful fluorescent intercalating dye that labels double-stranded DNA (dsDNA) [28]. The real-time PCR was used with the following composition: 1 × buffer, 2.5 mmol/L $MgCl_2$, 60 nmol/L SYBR-Green (Biozym) 1 mmol/L deoxynucleoside triphosphates (dNTPs), 1 mmol/L primer, 100 ng cDNA, 1 U Taq-Polymerase (Invitrogen). Specific primers were designed from the rat cDNA-sequences of $P2X_7$ receptor (sense 5′-GTGCCATTCTGACCAGGGTTGTATAAA-3′; antisense 5′-GCCACCTCTGTAAAGTTCTCTCCGATT-3′; and the housekeeping gene β-actin sense 5′-ACC TTCAACACCCCAGCCATGTACG-3′; and antisense 5′-CTGATCCACATCTGCTGGAAGGTGG-3′). The expected lengths of the PCR products were 353 bp and 697 bp, respectively. The cycle profile was 6 minutes at 94°C followed by 40 times 30 seconds at 94°C, annealing for either 58°C for the $P2X_7$ receptor or 65°C for β-actin and a 72°C extension for 1 minute.

For continuous monitoring, fluorescence emission was recorded after each cycle and for every reaction a threshold cycle (c_t) was evaluated by the Icycler Software (BioRad). To quantify $P2X_7$ receptor and β-actin gene expression, standard curves were generated with known amounts of each gene product.

Immunoblotting

Twenty nanograms of glomerular protein were mixed with loading buffer and incubated for 5 minutes at 95°C before being loaded on 12% SDS-polyacrylamide gel electrophoresis (PAGE). A prestained standard marker (BioRad, UK) was used to estimate molecular weight. The proteins were transferred semidry for 2 hours onto a Hybond enhanced chemiluminescence (ECL)-nitrocellulose membrane (Amersham, UK). The nitrocellulose was blocked (PBS containing 3% milk powder and 0.05% Tween 20) at room temperature and incubated overnight in the same solution containing 2.5 μg/mL antibody at 4°C. For detection, the ECL+ chemiluminescence method was performed using a peroxidase-linked donkey antirabbit IgG and ECL Western blotting reagents (Amersham, UK); light emission was captured by Multi-Imager scanning software (Bio-Rad).

Statistical analysis

All data are given as mean ± SEM. Differences between means were tested for significance by unpaired *t* test (Student). A P value of <0.05 was considered significant.

RESULTS

Immunohistochemistry

$P2X_7$ receptor immunoreactivity was barely detectable in normal rat kidney (Fig. 1A). In contrast, $P2X_7$ receptor immunoreactivity was clearly visible in glomeruli of kidney tissue from both STZ-induced diabetic (Fig. 1B) and TGR hypertensive rats (Fig. 1C).

Figure 2A to F shows $P2X_7$ receptor (red) immunofluorescence images of glomeruli of 6-week diabetic (Fig. 2A), control (Fig. 2B), and 12-week TGR hypertensive (Fig. 2C) rats, again showing increased $P2X_7$ receptor protein expression in the diabetic and hypertensive rat kidneys compared with control. Figure 2D shows $P2X_7$ receptor peptide pre-absorption in a 6-week diabetic kidney. Figure 2E and F show co-localization with a podocyte marker (Fig. 2E) [WT-1 (red)], but not a mesangial cell marker (Fig. 2F) [Thy-1 (green)] in 6-week diabetic kidney. Although increased $P2X_7$ receptor glomerular immunostaining was detectable in 3-week diabetic rats, there was no apparent further increase between weeks 6 and 9.

Immunoelectron microscopy localization of $P2X_7$ receptor

The electron microscopy images of diabetic animals localized immunoreactivity for $P2X_7$ receptor protein to podocytes (predominantly), rather than mesangial cells (not shown); minimal immunoprecipitation could be detected in healthy controls (Fig. 3A) compared with diabetic animals (Fig. 3B and C). In podocytes, immunoreactivity appeared as small clumps of immunoprecipitate in both the cytoplasm and on the cell membrane (Fig. 3B). The cell membrane labeling was particularly noted on some podocyte foot processes, at the glomerular filtration barrier (Fig. 3C). No immunolabeling was observed in diabetic tissue when the primary antibody was replaced with nonimmune serum (Fig. 3D).

TUNEL staining

As a marker of apoptosis, although we could demonstrate an increase in TUNEL staining in diabetic glomeruli (as shown previously by others [29]), and that the pattern looked similar to the glomerular $P2X_7$ receptor staining (compare Fig. 4 with Fig. 2A and C), for technical reasons we could not confidently establish co-localization of $P2X_7$ receptor immunostaining with TUNEL-positive cells.

Semiquantitative and quantitative (real-time) reverse transcription (RT)-PCR in diabetic kidney

The PCR produces single bands at 353 bp and 697 bp for the $P2X_7$ receptor and the housekeeper β-actin, respectively. Control and diabetic glomerular cDNA were standardized to β-actin and densiometric analysis showed

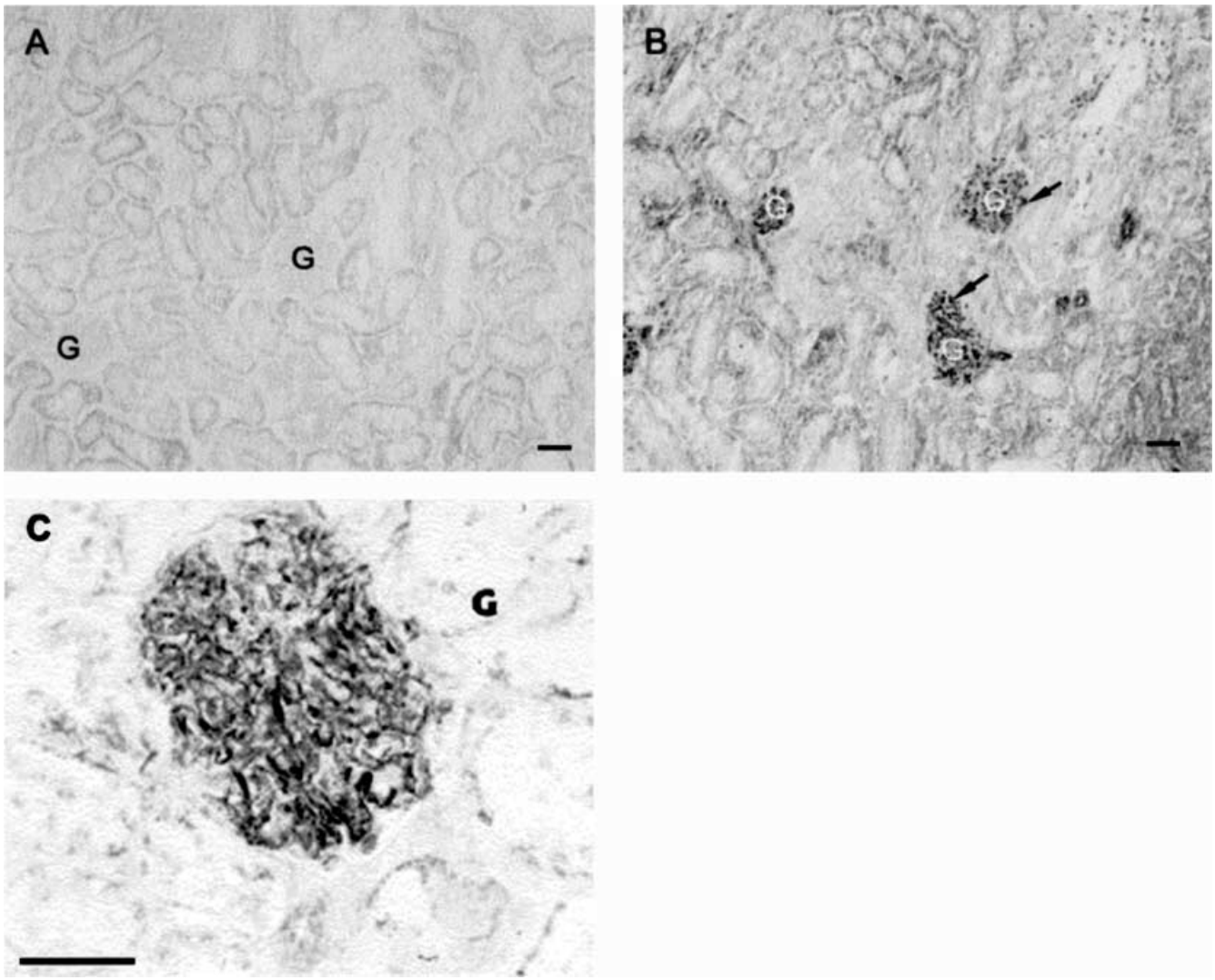

Fig. 1. Light micrographs. (*A*) Control rat kidney cortex showing minimal $P2X_7$ receptor immunoreactivity in tubules and glomeruli (G). (*B*) A 9-week diabetic kidney showing increased $P2X_7$ receptor immunoreactivity (arrows) in glomeruli (G). (*C*) A $P2X_7$ immunopositive glomerulus (G) of a 12-week Ren-2 transgenic (TGR) hypertensive rat (black scale bar = 50 μm).

no difference between the two groups on an agarose gel (Fig. 5). PCRs performed with the same amount of cDNA template, but with $P2X_7$ receptor primers, demonstrate an increase in mRNA abundance in glomeruli of diabetic animals after 6 weeks (Fig. 5). Experiments on 3-week diabetic animals could not detect any difference from controls (data not shown). Figure 6 shows the real-time PCR data expressed as a ratio of $P2X_7$ receptor to β-actin concentrations, which is increased in diabetic glomeruli.

Western blotting

Western blots incubated with $P2X_7$ receptor antibody produced clear bands at ~80 and ~50 kD with 20 μg of glomerular protein: a modest increase in $P2X_7$ receptor protein expression could be detected in 9-week diabetic glomeruli compared with control (Fig. 7A). Preincubation of the antibody with $P2X_7$ receptor peptide absorbed the bands completely (Fig. 7B). There was no detectable difference in $P2X_7$ receptor protein expression comparing 3-week and 6-week diabetic glomerular extracts with control (not shown).

DISCUSSION

Until recently, the P2X subfamily of ion channel receptors was considered exclusive to excitable tissues, but is now increasingly recognized as widespread in distribution [5]. Interest in studying the distinct family member $P2X_7$ derives from its unique structure (C-terminal extension) and unusual function (pore formation) [5]. This receptor was first cloned from rat brain [6] and subsequently from human monocytes [30] and mouse microglial cells [31]. Expression of its mRNA, detected by Northern blot analysis, was initially found (in addition to rat brain) in bone marrow, and tissues containing abundant macrophages

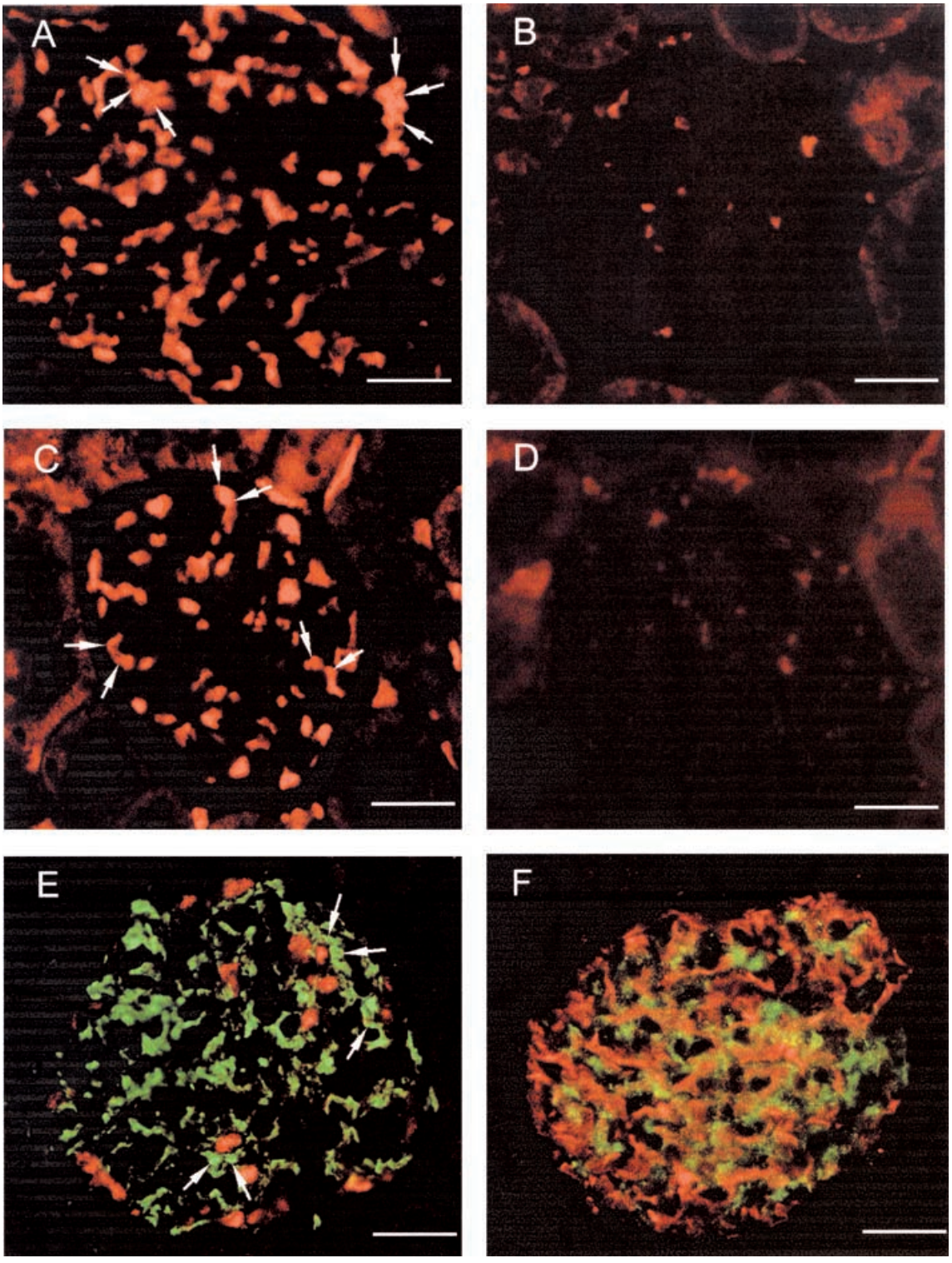

Fig. 2. Immunofluorescence photomicrographs. (*A*) Increased expression of $P2X_7$ receptor immunoreactivity (red) in a 6-week diabetic rat glomerulus. (*B*) Control glomerulus, which again shows little or no $P2X_7$ receptor immunoreactivity. (*C*) Increased expression of $P2X_7$ receptor immunoreactivity (red) in a 12-week Ren-2 transgenic (TGR) hypertensive rat glomerulus. (*D*) A diabetic glomerulus preabsorbed with excess $P2X_7$ receptor peptide showing no immunostaining. (*E*) $P2X_7$ receptor immunoreactivity (green) on podocytes co-localized with the podocyte nuclear protein Wilm's tumor (WT-1) (red). (*F*) Minimal co-localization of $P2X_7$ receptor (red) with the mesangial cell marker Thy-1 (green) (white scale bar = 30 μm).

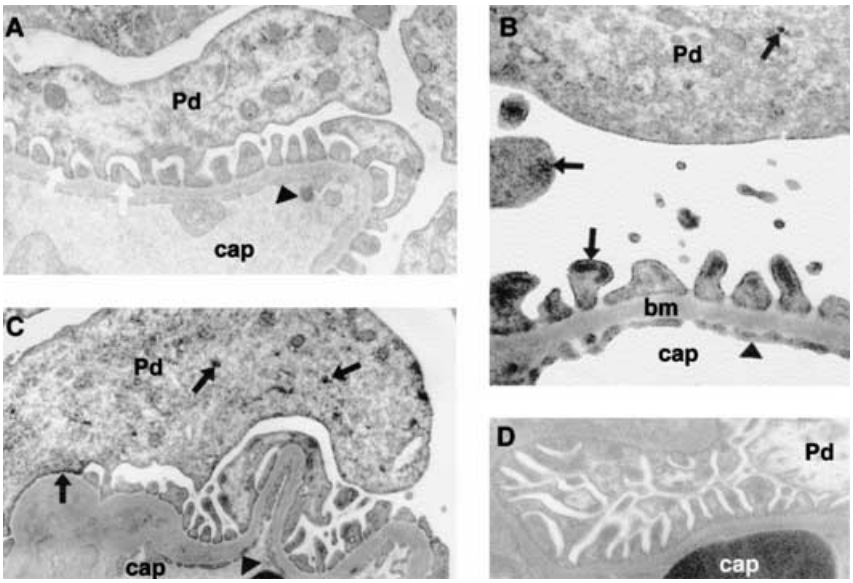

Fig. 3. Electron-immunocytochemistry of the $P2X_7$ receptor subtype in glomeruli. (*A*) Control, showing no $P2X_7$ receptor staining of podocytes (Pd), podocyte foot processes (white arrows), and fenestrated endothelium (black arrow head) of glomerular capillaries (cap) (magnification ×16,000). (*B*) $P2X_7$ receptor immunoprecipitate localized intracellularly and on the membrane of podocyte (Pd) foot processes (black arrows). Glomerular capillary (cap), basement membrane (bm), fenestrated endothelium (black arrow head) of a 3-week diabetic glomerulus (magnification ×15,000). (*C*) A higher magnification of the glomerular filtration barrier showing $P2X_7$ receptor immunoprecipitate intracellularly and in association with the cell membrane of secondary foot processes (black arrows) of a 9-week diabetic glomerulus. Fenestrated endothelium (black arrow head), lumen of capillary (cap) (magnification ×28,000). (*D*) An immunocytochemical control showing no immunoprecipitate when the $P2X_7$ receptor antibody was replaced with nonimmune 10% normal horse serum (magnification ×15,000).

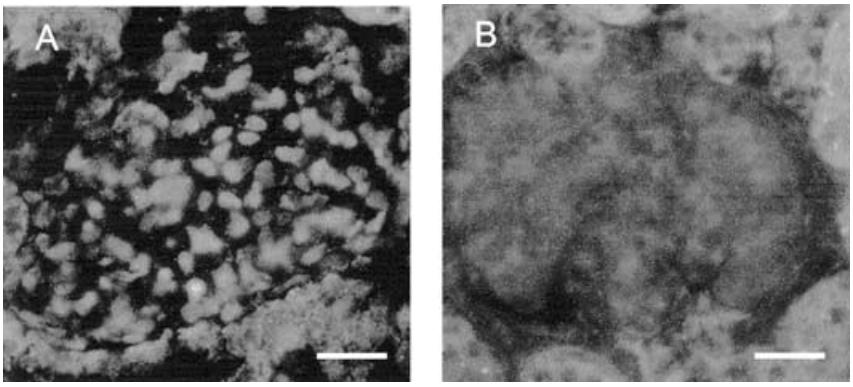

Fig. 4. Photomicrographs. (*A*) An increase in terminal deoxynucleotidyl transferase-mediated nick end-labeling (TUNEL)-positive cells in the glomerulus of a 9-week diabetic rat. (*B*) Comparison with a control glomerulus (scale bar = 30 μm).

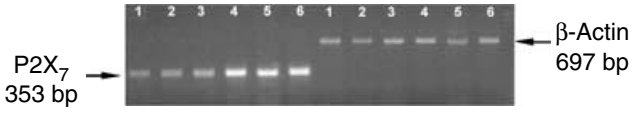

Fig. 5. Sample agarose gel from reverse transcription-polymerase chain reaction (RT-PCR) experiments. Lanes 1 to 3 are mRNA isolated from glomeruli of three control animals; lanes 4 to 6 are mRNA isolated from glomeruli of three 6-week diabetic animals. Levels of β-actin mRNA expression are unchanged; however there is an increase in expression of $P2X_7$ receptor mRNA in diabetic glomeruli.

or monocytes, but not in the kidney [32]. Later we, and others, found evidence for $P2X_7$ receptor expression in cultured mesangial cells and showed that it can mediate ATP-induced cell death by apoptosis [11, 12]. These observations prompted us to investigate whether $P2X_7$ receptor expression might be altered in two models of chronic glomerular injury: the STZ-induced model of diabetes and the transgenic (mRen2)27 renin-related model of severe hypertension.

Cell permeation by ATP was first reported in mast cells [7] (associated with degranulation and histamine release) and later in other hemopoietic cells, especially macrophages and monocytes; it is mediated by the $P2X_7$ receptor, formerly known as P2Z [5]. The ability of extracellular ATP to induce cell death is now well-established [33]; cells that express the $P2X_7$ receptor, such as macrophages and lymphocytes, can die by either apoptosis or necrosis [6, 34]. Although its physiologic function is still unclear, $P2X_7$ receptor expression in shedding epithelia, such as skin [22], duodenum [35], vagina and uterus [36], suggests that it might also have a role in normal cell turnover. It is an unusual receptor, because of its dual function as a rapidly opening ligand-gated ion channel and a slowly forming membrane pore [6, 30]; it can also cause cell fusion [37], membrane blebbing [38], and shedding of microvesicles (ectocytosis), which may bind cytokines or complement, as well as act as stimuli to cytokine release when phagocytically ingested and cleared [39]. Some authors have suggested that its ion channel

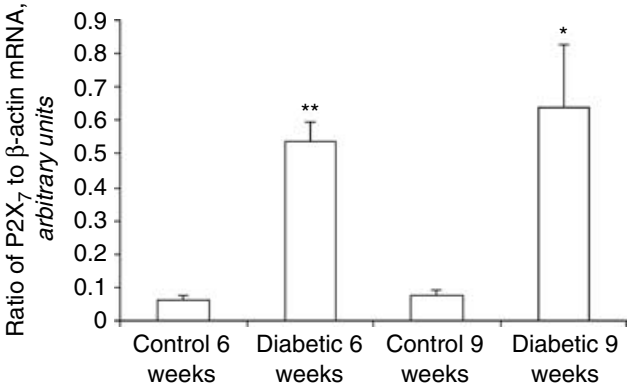

Fig. 6. Comparison of $P2X_7$ receptor mRNA levels by real time polymerase chain reaction (PCR) analysis expressed as a ratio to β-actin. An eightfold increase in $P2X_7$ receptor mRNA levels in 6-week diabetic glomeruli ($^{**}P < 0.005$), and in 9-week diabetic glomeruli ($^{*}P < 0.05$) when compared with control.

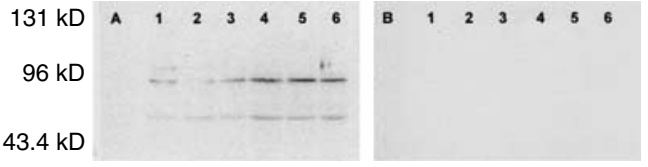

Fig. 7. Sample immunoblot using polyclonal $P2X_7$ receptor antibody. Lanes 1 to 3 are protein extracts from control glomeruli; lanes 4 to 6 are protein extracts from 9-week diabetic glomeruli. Twenty micrograms of glomerular protein were loaded onto each lane and β-actin was used as loading control (data not shown). (*A*) Increased $P2X_7$ receptor protein in diabetic glomeruli (lanes 4 to 6) compared with control (lanes 1 to 3). Two bands were detected, one of ~80 kD and another of ~50 kD. The larger band is probably the glycosylated monomeric form of the $P2X_7$ receptor [24] and the smaller band may be a degradation product. (*B*) Preabsorption of $P2X_7$ receptor antibody with homologous peptide completely abolished both protein bands.

activity versus pore formation might account for induction of apoptosis rather than necrosis, depending on cell type [34], and that these diverse, though related, responses might also depend on a difference in sensitivity to ATP. However, the level of receptor expression at the plasma membrane, together with the extent and duration of exposure to ATP are probably key determinants of the final response. While formation of the large plasma membrane pore is likely to result in cell death by lysis and necrosis, activation of the ligand-gated ion channel may lead to caspase activation and apoptosis after more prolonged exposure to ATP, or repeated $P2X_7$ receptor stimulation [6, 40].

In the context of tissue injury and inflammation, $P2X_7$ receptor activation has been shown to induce interleukin-converting enzyme (ICE) activity and release of mature interleukin-1β (IL-1β) from macrophages and microglial cells (perhaps by a $[K^+]_i$-dependent mechanism), which may itself be linked to activation of other caspases and therefore to apoptosis [34, 38, 40]. Lipopolysaccharide (LPS) and cytokines such as interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) can increase expression of the $P2X_7$ receptor and synergize with ATP to augment IL-1β release [11, 34]; moreover extracellular ATP can itself stimulate TNF-α release [41]. Recent data also show that stimulation of $P2X_7$ receptors by ATP activates the transcription factor nuclear factor-κB (NF-κB) [42], which is involved in regulating inflammatory cytokine synthesis; again potentially linking $P2X_7$ receptor stimulation to autocrine- or paracrine-induced apoptosis. Thus ATP, via $P2X_7$ receptors, could interact with, and orchestrate, inflammatory cytokines and the inflammatory response in vivo, eventually leading to cell death by necrosis or apoptosis; at least partially confirmed by a recent study of a $P2X_7$ knockout mouse [43]. In keeping with such a role, and in contrast to the P2Y receptor subfamily (and other P2X receptor subtypes), $P2X_7$ receptor stimulation requires relatively high concentrations of extracellular ATP (>100 μmol/L) [5], which are most likely to be found during platelet aggregation, thrombosis, and cell injury.

As already mentioned, in the kidney, the level of $P2X_7$ receptor mRNA is low or undetectable, at least in native tissue [30]. However, it has been more readily detectable in isolated and cultured rat glomerular mesangial cells [11, 12], mouse podocytes [13], and medullary collecting duct cells [44]. The intriguing observation from the present study is the strong (and seemingly unique) appearance and increase in $P2X_7$ receptor expression in the glomeruli of our two models of renal injury. Moreover, expression based on our electron microscopy findings was predominantly located to podocytes, although there was some staining of endothelial and mesangial cells. We have previously demonstrated electron microsopic immunolocalization of $P2X_7$ receptors in a subpopulation of unstimulated rat mesangial cells in culture [11], though immunoreactivity in these cells appeared to be more prevalent than in mesangial cells in situ, suggesting that cultured mesangial cells produce more $P2X_7$ receptor protein than their counterpart in native tissue and that there may be some heterogeneity of expression.

We can only speculate at present as to the likely role of the $P2X_7$ receptor. We have nothing with which to compare our observation of a localized increase in its expression, except with in vitro observations that bear some similarity. For example, altered $P2X_7$ receptor expression and increased sensitivity to ATP-induced apoptosis have been reported in cultured fibroblasts exposed to high concentrations of extracellular glucose [14]. In these cells $P2X_7$ receptor expression has also been linked to release of the autocrine growth factor IL-6 [45], which is increased in diabetic glomeruli [46]. In addition, IL-1β and TNF-α, both closely associated with $P2X_7$ receptor stimulation, are also increased in diabetic glomeruli [47]. A recent study by Rost et al [48] suggested that nucleotides might be involved in glomerular repair

following injury: they showed that the P2 receptor antagonist PPADS inhibited mesangial cell proliferation in the anti-Thy-1 model of proliferative glomerulonephritis. Although these authors presented data in support of an effect via a P2Y, rather than a P2X receptor, receptor characterization is limited because PPADS is generally regarded as a nonselective P2 receptor antagonist [49] that can also be active at the $P2X_7$ receptor [6] (a receptor not examined in their study), as well as being an inhibitor of ectoATPase and hence ATP breakdown [50].

Normally in the kidney, apoptosis occurs at a low level, but increases following various forms of injury, including ischemia [51] and in diabetes [29]. However, in some models healing follows this process [52], whereas in others it may lead to renal scarring [51]; therefore, regulation of this process could be important in normal tissue repair and remodeling following injury. Thus our finding of increased intensity of $P2X_7$ receptor immunoreactivity in the glomeruli of 12-week TGR and 6-week diabetic rats compared with their age-matched normal control animals might indicate a role for the $P2X_7$ receptor in glomerular repair by deleting damaged cells (although we could not unequivocally co-localize $P2X_7$ receptor expression with apoptotic cells), yet at the same time encouraging proliferation and repair. Although the implication that hypertensive damage might be an immune cell-mediated "inflammatory" process is indeed arguable, it is not without precedent [abstract; Collidge et al, *J Am Soc Nephrol* 13:53A, 2002]. An amelioration of both blood pressure and hypertensive intrarenal arterial damage in TGR rats treated with tacrolimus has been reported. What determines this balance will undoubtedly depend on the presence and expression of additional factors, including other P2 receptors, such as $P2Y_2$ [11, 26, 48], $P2Y_6$ [48] or $P2Y_1$ [26], as well as the activity of ATP degrading ectoATPases [53]. Moreover, the intracellular immunolocalization of the $P2X_7$ receptor seen in the present study, and also in our previous work on cultured mesangial cells [11], suggests the presence of a preformed receptor, which may only be expressed at the plasma membrane (for activation) following an as yet unknown stimulus. Further studies, perhaps in $P2X_7$ receptor knockout mice, are needed to confirm and extend these observations, and to determine their relationship to apoptosis (and diabetes) and if manipulation of the $P2X_7$ receptor is a potentially useful therapeutic option.

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REFERENCES

1. Burnstock G: The past, present and future of purine nucleotides as signalling molecules. *Neuropharmacol* 36:1127–1139, 1997
2. Burnstock G: A basis for distinguishing two types of purinergic receptor, in *Cell Membrane Receptor for Drugs and Hormones: A Multidisciplinary Approach*, edited by Straub RW, Bolis L, New York, Raven Press, 1978, pp 107–118
3. King BF, Burnstock G: Purinergic receptors (chapter 21), in *Understanding G Protein-Coupled Receptors and Their Role in the CNS*, edited by Pangalos MN, Davies CH, Oxford, Oxford University Press, 2002, pp 422–438
4. Abbracchio MP, Boeynaems J-M, Barnard EA, *et al*: Characterization of the UDP-glucose receptor (re-named here the $P2Y_{14}$ receptor) adds diversity to the P2Y receptor family. *Trends Pharmacol Sci* 24:52–55, 2003
5. North RA: Molecular physiology of P2X receptors. *Physiol Rev* 82:1013–1067, 2002
6. Surprenant A, Rassendren F, Kawashima E, *et al*: The cytolytic P2Z receptor for extracellular ATP identified as a P2X receptor ($P2X_7$). *Science* 272:735–738, 1996
7. Cockcroft S, Gomperts BD: ATP induces nucleotide permeability in rat mast cells. *Nature* 279:541–542, 1979
8. Baricordi OR, Melchiorri L, Adinolfi E, *et al*: Increased proliferation rate of lymphoid cells transfected with the P2X(7) ATP receptor. *J Biol Chem* 274:33206–33208, 1999
9. Di Virgilio F, Wiley JS: The $P2X_7$ receptor of CLL lymphocytes—A molecule with a split personality. *Lancet* 360:1898–1899, 2002
10. Li CM, Campbell SJ, Kumararatne DS, *et al*: Association of a polymorphism in the P2X7 gene with tuberculosis in a Gambian population. *J Infect Dis* 186:1458–1462, 2002
11. Harada H, Chan CM, Loesch A, *et al*: Induction of proliferation and apoptotic cell death via P2Y and P2X receptors, respectively, in rat glomerular mesangial cells. *Kidney Int* 57:949–958, 2000
12. Schulze-Lohoff E, Hugo C, Rost S, *et al*: Extracellular ATP causes apoptosis and necrosis of cultured mesangial cells via P2Z/P2X7 receptors. *Am J Physiol* 275:F962–F971, 1998
13. Fischer KG, Saueressig U, Jacobshagen C, *et al*: Extracellular nucleotides regulate cellular functions of podocytes in culture. *Am J Physiol* 281:F1075–F1081, 2001
14. Solini A, Chiozzi P, Falzoni S, *et al*: High glucose modulates $P2X_7$ receptor-mediated function in human primary fibroblasts. *Diabetologia* 43:1248–1256, 2000
15. Debnam ES, Ebrahim HY, Swaine DJ: Diabetes mellitus and sugar transport across the brush-border and basolateral membranes of rat jejunal enterocytes. *J Physiol* 424:13–25, 1990
16. Vallon V, Wead LM, Blantz RC: Renal hemodynamics and plasma and kidney angiotensin II in established diabetes mellitus in rats: Effect of sodium and salt restriction. *J Am Soc Nephrol* 5:1761–1767, 1995
17. Reyes AA, Karl IE, Kissane J, *et al*: L-arginine administration prevents glomerular hyperfiltration and decreases proteinuria in diabetic rats. *J Am Soc Nephrol* 4:1039–1045, 1993
18. Hicks KK, Seifen E, Stimers JR, *et al*: Effects of streptozotocin-induced diabetes on heart rate, blood pressure and cardiac autonomic nervous control. *J Auton Nerv Syst* 69:21–30, 1998
19. Mullins JJ, Peters J, Ganten D: Fulminant hypertension in transgenic rats harbouring the mouse Ren-2 gene. *Nature* 344:541–544, 1990
20. Springate JE, Feld LG, Ganten D: Renal function in hypertensive rats transgenic for mouse renin gene. *Am J Physiol* 266:F731–F737, 1994
21. Kelly DJ, Wilkinson-Berka JL, Allen TJ, *et al*: A new model of diabetic nephropathy with progressive renal impairment in

the transgenic (mRen-2)27 rat (TGR). *Kidney Int* 54:343–352, 1998
22. Gröschel-Stewart U, Bardini M, Robson T, *et al*: Localisation of $P2X_5$ and P2X7 receptors by immunohistochemistry in rat stratified squamous epithelia. *Cell Tissue Res* 296:599–605, 1999
23. Oglesby IB, Lachnit G, Burnstock G, *et al*: Subunit specificity of polyclonal antisera to the carboxy terminal regions of P2X receptors, $P2X_1$ through $P2X_7$. *Drug Dev Res* 47:189–195, 1999
24. Kim M, Spelta V, Sim J, *et al*: Differential assembly of rat purinergic $P2X_7$ receptor in immune cells of the brain and periphery. *J Biol Chem* 276:23262–23267, 2001
25. Mehta VB, Hart J, Wewers MD: ATP-stimulated release of interleukin (IL)-1β and IL-18 requires priming by lipopolysaccharide and is independent of caspase-1 cleavage. *J Biol Chem* 276:3820–3826, 2001
26. Turner CM, Vonend O, Chan C, *et al*: The pattern of distribution of selected ATP-sensitive P2 receptor subtypes in normal rat kidney: An immunohistological study. *Cells Tissues Organs* 175:105–117, 2003
27. Loesch A, Burnstock G: Electron-immunocytochemical localization of $P2X_1$ receptors in the rat cerebellum. *Cell Tissue Res* 294:253–260, 1998
28. Wittwer CT, Herrmann MG, Moss AA, *et al*: Continuous fluorescence monitoring of rapid cycle DNA amplification. *Biotechniques* 22:130–138, 1997
29. Pesce C, Menini S, Pricci F, *et al*: Glomerular cell replication and cell loss through apoptosis in experimental diabetes mellitus. *Nephron* 90:484–488, 2002
30. Rassendren F, Buell GN, Virginio C, *et al*: The permeabilizing ATP receptor, P2X7. Cloning and expression of a human cDNA. *J Biol Chem* 272:5482–5486, 1997
31. Chessell IP, Simon J, Hibell AD, *et al*: Cloning and functional characterisation of the mouse $P2X_7$ receptor. *FEBS Lett* 439:26–30, 1998
32. Collo G, Neidhart S, Kawashima E, *et al*: Tissue distribution of the $P2X_7$ receptor. *Neuropharmacol* 36:1277–1283, 1997
33. Chow SC, Kass GE, Orrenius S: Purines and their roles in apoptosis. *Neuropharmacol* 36:1149–1156, 1997
34. Di Virgilio F, Chiozzi P, Falzoni S, *et al*: Cytolytic P2X purinoceptors. *Cell Death Differ* 5:191–199, 1998
35. Gröschel-Stewart U, Bardini M, Robson T, *et al*: P2X receptors in the rat duodenal villus. *Cell Tissue Res* 297:111–117, 1999
36. Bardini M, Lee HY, Burnstock G: Distribution of P2X receptor subtypes in the rat female reproductive tract at late prooestrus/early oestrus. *Cell Tissue Res* 299:105–113, 2000
37. Chiozzi P, Sanz JM, Ferrari D, *et al*: Spontaneous cell fusion in macrophage cultures expressing high levels of the P2Z/$P2X_7$ receptor. *J Cell Biol* 138:697–706, 1997
38. Mackenzie A, Wilson HL, Kiss-Toth E, *et al*: Rapid secretion of interleukin-1β by microvesicle shedding. *Immunity* 15:825–835, 2001
39. Schifferli JA, Sadallah S, Gasser O, *et al*: Auto-anticorps anti-C1q dans la maladie lupique, in *Actualites Nephrologiques Jean Hamburger Hopital Necker*, edited by Grunfeld J-P, Paris, Medecine-Sciences Flammarion, 2003, pp 103–115
40. Ferrari D, Los M, Bauer MK, *et al*: P2Z purinoreceptor ligation induces activation of caspases with distinct roles in apoptotic and necrotic alterations of cell death. *FEBS Lett* 447:71–75, 1999
41. Tonetti M, Sturla L, Giovine M, *et al*: Extracellular ATP enhances mRNA levels of nitric oxide synthase and TNFα in lipopolysaccharide-treated RAW 264.7 murine macrophages. *Biochem Biophys Res Commun* 214:125–130, 1995
42. Ferrari D, Wesselborg S, Bauer MK, *et al*: Extracellular ATP activates transcription factor NF-kappaB through the P2Z purinoreceptor by selectively targeting NF-kappaB p65. *J Cell Biol* 139:1635–1643, 1997
43. Solle M, Labasi J, Perregaux DG, *et al*: Altered cytokine production in mice lacking P2X(7) receptors. *J Biol Chem* 276:125–132, 2001
44. Schwiebert EM, Kishore BK: Extracellular nucleotide signaling along the renal epithelium. *Am J Physiol* 280:F945–F963, 2001
45. Solini A, Chiozzi P, Morelli A, *et al*: Human primary fibroblasts in vitro express a purinergic P2X7 receptor coupled to ion fluxes, microvesicle formation and IL-6 release. *J Cell Sci* 112:297–305, 1999
46. Suzuki D, Miyazaki M, Naka R, *et al*: In situ hybridization of interleukin 6 in diabetic nephropathy. *Diabetes* 44:1233–1238, 1995
47. Hasegawa G, Nakano K, Sawada M, *et al*: Possible role of tumor necrosis factor and interleukin-1 in the development of diabetic nephropathy. *Kidney Int* 40:1007–1012, 1991
48. Rost S, Daniel C, Schulze-Lohoff E, *et al*: P2 receptor antagonist PPADS inhibits mesangial cell proliferation in experimental mesangial proliferative glomerulonephritis. *Kidney Int* 62:1659–1671, 2002
49. Khakh BS, Burnstock G, Kennedy C, *et al*: International union of pharmacology. XXIV. Current status of the nomenclature and properties of P2X receptors and their subunits. *Pharmacol Rev* 53:107–118, 2001
50. Chen BC, Lee CM, Lin WW: Inhibition of ecto-ATPase by PPADS, suramin and reactive blue in endothelial cells, C6 glioma cells and RAW 264.7 macrophages. *Br J Pharmacol* 119:1628–1634, 1996
51. Savill J, Mooney A, Hughes J: What role does apoptosis play in progression of renal disease? *Curr Opin Nephrol Hypertens* 5:369–374, 1996
52. Shimizu A, Kitamura H, Masuda Y, *et al*: Apoptosis in the repair process of experimental proliferative glomerulonephritis. *Kidney Int* 47:114–121, 1995
53. Poelstra K, Heynen ER, Baller JF, *et al*: Modulation of anti-Thy1 nephritis in the rat by adenine nucleotides. Evidence for an anti-inflammatory role for nucleotidases. *Lab Invest* 66:555–563, 1992