EBioMedicine xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

EBioMedicine

journal homepage: www.ebiomedicine.com

Research Paper

Hyperglycemia-induced Renal P2X7 Receptor Activation Enhances Diabetes-related Injury

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ARTICLE INFO

Article history:
Received 26 February 2017
Received in revised form 31 March 2017
Accepted 6 April 2017
Available online xxxx

Keywords:
Glucose
Purine
P2
Renal
Cytokine
CKD

ABSTRACT

Diabetes is a leading cause of renal disease. Glomerular mesangial expansion and fibrosis are hallmarks of diabetic nephropathy and this is thought to be promoted by infiltration of circulating macrophages. Monocyte chemoattractant protein-1 (MCP-1) has been shown to attract macrophages in kidney diseases. P2X7 receptors (P2X7R) are highly expressed on macrophages and are essential components of pro-inflammatory signaling in multiple tissues. Here we show that in diabetic patients, renal P2X7R expression is associated with severe mesangial expansion, impaired glomerular filtration (≤40 ml/min/1.73 sq. m.), and increased interstitial fibrosis. P2X7R activation enhanced the release of MCP-1 in human mesangial cells cultured under high glucose conditions. In mice, P2X7R-deficiency prevented glomerular macrophage attraction and collagen IV deposition; however, the more severe interstitial inflammation and fibrosis often seen in human diabetic kidney diseases was not modelled. Finally, we demonstrate that a P2X7R inhibitor (AZ11657312) can reduce renal macrophage accrual following the establishment of hyperglycemia in a model of diabetic nephropathy. Collectively these data suggest that P2X7R activation may contribute to the high prevalence of kidney disease found in diabetics.

1. Introduction

Diabetic kidney disease is becoming a global epidemic (Gross et al., 2005) and is expected to increase in prevalence as the diabetic population grows (Shaw et al., 2010). Diabetic nephropathy is a unique predictor of mortality in both insulin-dependent and -independent diabetes (Afkarian et al., 2013; Groop et al., 2009). Antihypertensive, antiproteinuric and blood-glucose lowering therapies are the mainstays of treatment. However, despite aggressive blood pressure management (Gross et al., 2005; Parving et al., 1983) and hyperglycemic control (The Diabetes Control and Complications Trial Research Group, 1993), most diabetic patients progress to chronic kidney disease (CKD) and eventually end-stage renal failure.

Diabetic nephropathy is driven by mobilization of major inflammatory cascades (Wada and Makino, 2013) that result in glomerular extracellular matrix (ECM) deposition, basement membrane thickening, mesangial sclerosis, proteinuria and interstitial fibrosis (Mason and Wahab, 2003; Tervaert et al., 2010). Pro-inflammatory signaling may begin in the glomerular mesangium (Gómez-Guerrero et al., 2005; Savill et al., 1992). Exposure to a high glucose *milieu* can enhance mesangial secretion of the monocyte chemoattractant protein-1 (MCP-1) resulting in macrophage accumulation and glomerulonephritis (Ihm et al., 1998; Wada et al., 2000), leading ultimately to fibrosis (Zeisberg and Neilson, 2010). In diabetic patients, the ratio of MCP-1 to creatinine is a predictor of reduced glomerular filtration rate (GFR) and renal impairment (Tam et al., 2009).

Multiple adenosine-5′-triphosphate (ATP)-activated (P2X) receptors and G-protein coupled purinergic (P2Y) receptors regulate MCP-1 secretion in cell models (Shieh et al., 2014; Stokes and Surprenant, 2007). There are seven P2X receptors that when activated by ATP, function as non-selective cation channels (Costa-Junior et al., 2011). The P2X7 receptor (P2X7R) has a uniquely long carboxyl domain that interacts with membrane pores such as pannexin-1 - to drive inflammation, cell death, and tissue remodelling (Lister et al., 2007; Menzies et al., 2015b). P2X7R is highly expressed on macrophages where it regulates the maturation and release of cytokines (Ferrari et al., 2006), and on mesangial cells where it promotes ECM expansion (Solini et al., 2014, 2005). P2X7R is also expressed on non-immune cells, including those

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http://dx.doi.org/10.1016/j.ebiom.2017.04.011

of the pancreas (Coutinho-Silva et al., 2007) and renal microvasculature (Menzies et al., 2013).

Preclinical research has identified P2X7R as an attractive therapeutic target in kidney diseases (Menzies et al., 2016; Solini et al., 2013). However, it is not yet clear whether P2X7R activation regulates human diabetic nephropathy or, specifically, if glomerular P2X7R activation underlies disease severity. In the present study P2X7R was immunolocalized in renal biopsies from diabetic patients and assessed against indicators of renal function and disease. Resident glomerular cells facilitate the accrual of macrophages and so P2X7R-mediated pro-inflammatory signaling was investigated in primary human mesangial cells exposed to high glucose. We next investigated whether P2X7R-deficiency protected against diabetic renal injury in a murine model. In a final experiment, we investigated the therapeutic potential of a P2X7R inhibitor (P2X7Ri) following the establishment of hyperglycemia in a rat model of diabetic nephropathy.

2. Materials and Methods

2.1. Human Biopsy Samples

Renal biopsy specimens were obtained from patients after informed consent (local ethics committee approval; NRES Committee London - West London & GTAC; 04/Q0406/25), and only materials surplus to clinical need were used. Thin basement membrane (TBM) biopsies served as controls for diabetic nephropathy. TBM represents the carrier state for autosomal recessive Alport Syndrome, in which there are mutations in basement membrane collagen typically without renal impairment (Savige et al., 2013). Paraffin sections were retrieved from the histology archive at Hammersmith Hospital for 3 TBM patients and 30 diabetics (19/30; 63% type II).

2.2. Immunohistochemistry

Primary sections were de-waxed through xylenes and graded alcohols to water. Heat-induced epitope retrieval (HIER) was used. Sections were placed in boiling sodium citrate buffer (0.01 M; pH 6.0) and transferred to a water bath (95 °C for 20 min). Sections were then cooled under running water for 5 min and placed in phosphate-buffered saline (PBS; Sigma-Aldrich, Poole, UK). Sections were incubated in 0.3% hydrogen peroxide in PBS (10 min) then washed in PBS 3 times for 5 min each. Sections were incubated in either 10% dry powdered milk (Marvel; Premier Foods, St Albans, UK) or 20% normal goat serum (Dako; Carpinteria, CA, USA) in PBS for 30 min at room temperature.

Primary antibodies for P2X7R (APR-004, Alomone, RRID: AB_2040068; 1:50 murine; 1:100 human), human CD68 (Clone KP-1, Dako, RRID: AB_563621; 1:100), murine CD68 (Clone FA-11, Serotec, RRID: AB_323909; 1:200) and collagen IV (Ab6586, Abcam, RRID: AB_305584; 1:100) were reconstituted in PBS. Blocking solution was tapped off the slide and residual solution blotted. Primary antibody was applied at sufficient volume to cover the section (typically 100–200 μl). Slides were incubated in a covered, humidified staining chamber for 1 h (or overnight for human P2X7R staining). Negative controls comprised omission of primary antibody, addition of polyclonal normal rabbit immunoglobulin at an equivalent concentration to the primary antibody, and use of primary antibody pre-incubated with the cognate immunizing peptide at a 1:1 ratio. Detection was performed by a commercial system (EnVision; Dako, Carpinteria, CA, USA). Positive staining appeared as brown deposits.

Glomerular macrophage (CD68+) number was counted for 20 glomeruli in each section and the mean value obtained. The same starting point was used in each section and the first 20 randomly encountered glomeruli were included. Interstitial macrophage number was counted for 5 randomly selected fields of view (×200 magnification) and the mean value obtained. Glomeruli were outlined and area measured and type IV collagen calculated as percentage stained area per glomerular area. Fifteen glomeruli in each section were scored and the mean value taken.

2.3. Primary Human Mesangial Cells

Primary human mesangial cells (pHMCs; Lonza Biologics, Slough, UK) were cultured in a proprietary basal medium supplemented with 5% fetal calf serum (FCS) and gentamicin amphotericin-B (Sigma-Aldrich, Poole, UK). Cells were grown at 37 °C in a humidified atmosphere of 5% CO_2. Medium was changed every 2–3 days. At 90–100% confluence cells were sub-cultured onto chamber slides (Nunc, Lab-Tek, Thermo Fisher Scientific, Waltham, MA, USA) or 96-well plates (Nunc, Maxisorp, Thermo Fisher Scientific). Viability of pHMCs cultured in the presence of small molecule receptor inhibitors was assessed using a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay (CellTiter 96; Promega, Madison, WI, USA).

For experiments cells were grown >70% confluence and then made quiescent by incubation in glucose-free RPMI 1640 supplemented with 4 mM D-glucose with no serum for 24 h to induce cell cycle synchronization and quiescence prior to treatment. All experiments were done in serum free medium. Supplementing glucose-free RPMI 1640 with 30 mM D-glucose simulated hyperglycemic conditions. L-glucose, the metabolically inactive stereoisomer of D-glucose, was added to 4 mM D-glucose media at a concentration of 26 mM to act as an osmotic control for hyperglycemic media. Cells were grown in these conditions for 48 h without a medium change, before collection of supernatants for MCP-1 quantification (below) or cell monolayer lysates for protein extraction.

The effects of a selective P2X7R inhibitor (A438079; http://www.guidetopharmacology.org/GRAC/LigandDisplayForward?tab=biology&ligandId=4118; Tocris Bioscience, Bristol, UK), a P2X7R agonist BzATP, (Tocris), on MCP-1 production were tested both in the presence and absence of hyperglycemia by supplementing 4 mM D-glucose and 30 mM D-glucose media respectively, with the appropriate reagent concentration. Cells were treated for 30 min before collecting supernatants.

2.4. Protein Extraction

Cells were washed twice with ice-cold PBS before adding 100 μl of a proprietary cell lysis buffer (Invitrogen; Constituents: 10 mM Tris pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM Na4P2O7, 2 mM Na3VO4, 1% Triton X-100, 10% glycerol, 0.1% SDS, 0.5% deoxycholate) supplemented with 1 mM phenylmethanesulfonylfluoride (PMSF; a serine protease inhibitor) and protease inhibitor cocktail (P2714, Sigma Aldrich, Poole, UK). Monolayers were agitated for 2–3 min before scraping and collecting the lysate into microcentrifuge tubes. Tubes were rested on ice for 30 min, vortexing at 10 minute intervals, to ensure complete lysis of cell membranes and solubilization of proteins. The extract was then centrifuged at 18,000 ×*g* for 10 min at 4°C to pellet insoluble cellular debris. Supernatant was transferred to a fresh microcentrifuge tube and protein concentration quantified (Pierce BCA kit, Thermo Fisher Scientific).

2.5. Immunofluorescent Staining of P2X7R in pHMC

Medium was removed and cells washed twice with PBS (Sigma-Aldrich, Poole, UK). Cells were fixed in 4% paraformaldehyde (PFA; Sigma-Aldrich, Poole, UK) in PBS for 5 min at room temperature and washed 3 times with PBS. Cells were permeabilized with 1 ml 0.1% Triton X-100 (Sigma-Aldrich) in PBS for 10 min and washed twice with PBS.

Non-specific binding sites were blocked by incubation with 5% BSA in 0.1% Triton in PBS for 1 h at room temperature. Primary P2X7R antibody (Ab109246; Abcam, RRID: AB_10858498, Cambridge, UK) or

Please cite this article as: Menzies, R.I., et al., Hyperglycemia-induced Renal P2X7 Receptor Activation Enhances Diabetes-related Injury, EBioMedicine (2017), http://dx.doi.org/10.1016/j.ebiom.2017.04.011