The International Journal of Biochemistry & Cell Biology 45 (2013) 932–943

Contents lists available at SciVerse ScienceDirect

The International Journal of Biochemistry & Cell Biology

journal homepage: www.elsevier.com/locate/biocel

ATP-P2X4 signaling mediates NLRP3 inflammasome activation: A novel pathway of diabetic nephropathy

Kehong Chen [1], Jianguo Zhang [1], Weiwei Zhang, Jinhua Zhang, Jurong Yang, Kailong Li, Yani He *

Department of Nephrology, Daping Hospital, Research Institute of Surgery, Third Military Medical University, Chongqing 400042, PR China

ARTICLE INFO

Article history:
Received 27 November 2012
Received in revised form 6 February 2013
Accepted 11 February 2013
Available online 19 February 2013

Keywords:
P2X4
Extracellular ATP
NLRP3 inflammasome
Tubulointerstitial inflammation
Diabetic nephropathy

ABSTRACT

Tubulointerstitial inflammation plays a key role in the development of diabetic nephropathy (DN). Cytokines in the IL-1 family are the key pro-inflammatory cytokines of tubulointerstitial inflammation. Extracellular ATP can cause P2X receptors to activate the NOD-like receptor 3 (NLRP3) inflammasome and cause IL-1β and IL-18 maturation and release. We investigated the role of ATP-P2X4 signaling in NLRP3 inflammasome activation and renal interstitial inflammation characteristic of DN. Ex vivo studies, P2X4 showed increased expression in renal tubule epithelial cells in patients with nephropathy due to type 2 diabetes compared to those in the control group. Linear correlation analysis shows that P2X4 expression was positively related with urine IL-1β and IL-18 levels. Moreover, P2X4 expression was co-localized with NLRP3, IL-1β, and IL-18 expression. In vitro culture experiments showed NLRP3 protein expression, cleavage of caspase-1 and IL-1β, and release of IL-1β, IL-18 and ATP in HK-2 cells significantly increased after high glucose stimulation. However, apyrase, which consumes extracellular ATP, completely blocked the changes caused by high glucose. The P2 receptor antagonist suramin, P2X receptor antagonist TNP-ATP, P2X4 selective antagonist 5-BDBD, and P2X4 gene silencing attenuated NLRP3 expression, cleavage of caspase-1 and IL-1β, and release of IL-1β and IL-18 induced by high glucose. Taken together, these results suggest that ATP-P2X4 signaling mediates high glucose-induced activation of the NLRP3 inflammasome, regulates IL-1 family cytokine secretion, and causes the development of tubulointerstitial inflammation in DN.

1. Introduction

Diabetic nephropathy (DN) is the leading cause of end-stage renal disease (Collins et al., 2012). Tubulointerstitial inflammation is crucial in promoting the development and progression of DN. The NOD-like receptor 3 (NLRP3) inflammasome is activated in kidney tissue of streptozotocin-induced diabetic rats, and suppression of NLRP3 inflammasome activation can significantly reduce renal tissue inflammation and improve renal function (Wang et al., 2012). NLRP3 knock-out mice are protected from renal tubule damage and renal interstitial inflammation in kidney unilateral ureteral occlusion (UUO) and ischemia–reperfusion models (Shigeoka et al., 2010; Vilaysane et al., 2010). These data show that the NLRP3 inflammasome plays a key role in the process of kidney sterile inflammation. However, the mechanism of NLRP3 inflammasome activation in DN is still unclear.

A previous study reported that high glucose stimulated adenosine triphosphate (ATP) secretion by renal inherent cells (Solini et al., 2005). Increased extracellular ATP (eATP) activates the P2 receptors of immune cells (Trautmann, 2009) and initiates the immune reaction (Vitiello et al., 2012). Activation of P2 receptors causes the production of reactive oxygen species, chemokines and inflammatory markers, which consequently cause inflammation (Bours et al., 2011). It has been reported that eATP can activate the NLRP3 inflammasome and causes aseptic inflammation (Iyer et al., 2009).

Hyperglycemia can also cause retinal cell caspase-1 activation and interleukin (IL) -1β secretion in an eATP dependent manner (Trueblood et al., 2011). IL-1 family cytokines are pluripotent immunomodulatory cytokines that play a key role in tubulointerstitial inflammation in DN (Navarro-Gonzalez and Mora-Fernandez, 2008). They have direct relationships with many chronic complications of diabetes since they are able to induce production of various inflammatory factors (chemokines, metal proteases, tumor necrosis factor-α (TNF-α), etc.) and inflammatory cell infiltration (Devaraj et al., 2007; Vincent and Mohr, 2007). Pro-IL-1β and pro-IL-18, the precursor molecules of IL-1 family cytokines, rely on caspase-1 shearing action to exert their biological effects (Weber

* Corresponding author. Tel.: +86 23 68757871; fax: +86 23 68893060.
E-mail address: heynmail@yahoo.com (Y. He).
[1] Both authors contributed equally to the study.

1357-2725/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.biocel.2013.02.009

et al., 2010a). The NLRP3 inflammasome, the intracellular receptor of danger-associated molecular pattern molecules (DAMPs), can activate caspase-1, regulate IL-1β and IL-18 maturation and secretion, and trigger the inflammation of metabolic diseases such as diabetes, obesity, and gout (Liu-Bryan, 2010; Masters et al., 2010; Stienstra et al., 2011).

Activated purinergic P2X receptors mediate endogenous DAMPs such as ATP, biglycan, and amyloid protein (Babelova et al., 2009; Halle et al., 2008; Riteau et al., 2010), activate the NLRP3 inflammasome, and regulate aseptic inflammation in hypersensitivity (Weber et al., 2010b), acute pancreatitis (Hoque et al., 2011) and cancer (Ghiringhelli et al., 2009). P2X receptors are ligand-gated ion channel receptors in the P2 family. There are seven subtypes of P2X receptors (P2X1-7). All are expressed in the kidney except P2X3 (Turner et al., 2003). Renal tubule epithelial cells mainly express P2X4 and P2X6. P2X receptor activation can promote renal sodium/water discharge (Jankowski et al., 2011) and regulate renal blood flow (Crawford et al., 2011) in physiological conditions. The role of P2X4 in diabetic nephropathy is still undefined. The purpose of this study is to investigate the role of ATP-P2X4 signaling in NLRP3 inflammasome activation and renal interstitial inflammation of DN.

2. Materials and methods

2.1. Patients

A total of 45 patients with type 2 diabetic nephropathy were recruited for this study from the Department of Nephrology in Daping Hospital from January 1, 2011 to June 1, 2012. The enrollment criteria were as follows: patients were 40–70 years old with a history of type 2 diabetes; 24 h urine protein was above 150 mg; renal biopsy pathology led to a diagnosis of diabetic nephropathy; and no fever with obvious infection lesions or high uric acid. All patients used insulin to control blood glucose, angiotensin antagonist and CCB to control blood pressure, and statins to control lipids. Patients abstained from traditional Chinese medicine or sulfonylureas for three months after renal biopsy. Normal kidney tissues from nephrectomies of renal hamartoma were collected to be used in the control group. The protocol for this study was approved by the Ethical and Protocol Review Committee of the Third Military Medical University, and informed consent was obtained from the subjects.

2.2. Biochemical analysis

Blood and urine samples were collected 1 day prior to renal biopsy for biochemical analysis. Serum creatinine was measured by the modified Jaffé rate-blanked alkaline picrate method. The 24 h urinary protein excretion was measured by the benzethonium chloride method. Serum uric acid was determined by an enzymatic method. HbA_{1c} was calculated from total glycated hemoglobin, which was determined by the ion capture method; the normal range was <6.5%. The estimated glomerular filtration rate (eGFR) was calculated by the Cockcroft–Gault formula.

2.3. Immunohistochemistry analysis

The expression of P2X4 and NLRP3 proteins was determined by a 2-step immunohistochemical staining technique, as described previously (Liu et al., 2011). Specimens were deparaffinized and rehydrated. After antigen retrieval, polyclonal primary anti-P2X4 antibody (sc-28764, Santa Cruz Biotechnology, USA) and anti-NLRP3 antibody (ab4207, Abcam, UK), rabbit IgG isotype control antibody (ab27472, Abcam, UK) and goat IgG isotype control antibody (sc-3887, Santa Cruz Biotechnology, USA) were added and specimens were incubated at 4 °C overnight. IgG-conjugated horseradish peroxidase (HRP) and 3,3-diaminobenzidine tetrahydrochloride (ZLI-9032, Zhong Shan Golden Bridge Biological Technology, Beijing, China) were employed to visualize antibody binding.

Ten high power fields were randomly selected. Areas of brown nuclear staining for P2X4, NLRP3, IL-1β or IL-18 were counted and expressed as the percentage of total renal tubular epithelial cells (RTECs). The stained areas were rated as described previously (Liu et al., 2011): 0, no staining or positive staining in <10% of RTECs; 1, weak positive staining in 10% to 35% of RTECs; 2, moderate positive staining in 35–70% of RTECs; and 3, strong positive staining in >70% of RTECs. The counting of microscopic fields was performed by two blinded pathologists.

2.4. Confocal fluorescence analysis

Tissue sections were blocked and incubated with anti-P2X4 antibody or anti-NLRP3 antibody together with anti-IL-1β antibody (sc-52012, Santa Cruz Biotechnology, USA) or anti-IL-18 antibody (sc-133127, Santa Cruz Biotechnology, USA) at 4 °C overnight. After rinsing with PBS, the samples were stained with fluorescein isothiocyanate conjugated (A0562 or A0568, Beyotime, China) or Cy3-conjugated goat anti-mouse IgG (A0502 or A0521, Beyotime, China) for 60 min at 37 °C. Nuclei were stained with DAPI. The samples were mounted with glycerol and visualized under a confocal scanning microscope (Lcssp-2, Leica, Germany).

2.5. Cell culture and siRNA transfection

HK-2 cells (CRL-2190, American Type Culture Collection) were cultured in Dulbecco's modified Eagle's medium with low glucose (HyClone, USA). The cells were seeded at 1.5×10^6 cells/10 cm diameter dish for 2–3 days at 37 °C in a humidified atmosphere containing 5% CO_2. HK-2 cells were grown in media with normal glucose concentration (5 mM), or high glucose concentration (15, 25, 35, and 50 mM), or high mannitol concentrations (5 mM glucose + 30 mM mannitol) for 48 h. HK-2 cells were incubated in media with high glucose (35 mM, 48 h) with and without 5 U/ml apyrase (Sigma–Aldrich, USA), 100 μM suramin (Sigma–Aldrich, USA), 10 μM TNP-ATP (Sigma–Aldrich, USA), or 2 μM 5-BDBD (Tocris Bioscience, USA).

Transfection of siRNA was performed as previously described (Wu et al., 2010). Transfection control cells were transfected with siRNA for unrelated genes (fluorescein conjugated control siRNAs, sc-36869, Santa Cruz Biotechnology, USA) or control siRNA (sc-37007, Santa Cruz Biotechnology, USA), while experimental cells were transfected with siRNA directed against P2X4 (sequence: GTA CTA CAG AGA CCT GGCT), with all transfections utilizing Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Cells incubated with Lipofectamine 2000 were also used as a negative control. P2X4 knockdown was confirmed by Western blot and quantitative reverse transcription-PCR (qRT-PCR).

2.6. RNA Extraction and real-time quantitative PCR

Total RNA was isolated using RNAout (TOYOBO, Japan) according to the manufacturer's protocol, and P2X4 mRNA was evaluated by quantitative real-time PCR. The primers for P2X4 were designed based on GenBank sequences (accession numbers: NM_001256796.1) and synthesized (Sagon Inc., Shanghai, China). β-Actin was employed as an internal control, and its primer was designed based on the GenBank sequence (accession number for β-actin: NM_001017992.2). cDNAs for real-time quantitative PCR were synthesized using total RNAs from cell lysates. To avoid