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ORIGINAL ARTICLE

$P2X_7$ receptor antagonism attenuates the hypertension and renal injury in Dahl salt-sensitive rats

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The $P2X_7$ receptor is a ligand-gated ion channel activated by extracellular ATP, and a common genetic variation in the *$P2X_7$* gene significantly affects blood pressure. $P2X_7$ receptor expression is associated with renal injury and some inflammatory diseases. Brilliant blue G (BBG) is a selective rat $P2X_7$ receptor antagonist. In this study, to test whether BBG has protective effects on salt-sensitive hypertension and renal injury, Dahl salt-sensitive (DS) rats fed an 8% NaCl diet were i.p. injected with BBG (50 mg kg^{-1} per day) for 4 weeks. We also tested another $P2X_7$ receptor antagonist, namely A-438079 (100 mg kg^{-1} per day), for 7 days. We found that $P2X_7$ antagonism markedly attenuated salt-sensitive hypertension, urinary protein or albumin excretion, renal interstitial fibrosis and macrophage and T-cell infiltration in the DS rats, and significantly improved creatinine clearance. In an *in vitro* experiment using macrophages, we showed that lipopolysaccharide (LPS)-primed macrophages from the DS rats released more interleukin-1 beta in response to BzATP, a $P2X_7$ receptor agonist, than the macrophages from Lewis rats, possibly due to higher $P2X_7$ expression in the DS rats. In conclusion, *in vivo* blockade of $P2X_7$ receptors attenuated salt-sensitive hypertension and renal injury in the DS rats. Thus, $P2X_7$ appears to be responsible for a vicious cycle of salt-sensitive hypertension and renal injury in the DS rats, through higher expression in the immune cells. Furthermore, $P2X_7$ antagonists can prevent the development of salt-sensitive hypertension and renal injury, thus confirming that the $P2X_7$ receptor is an important therapeutic target.
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INTRODUCTION

The $P2X_7$ receptor is an adenosine-5′-triphosphate (ATP)-gated cation channel that is expressed mainly in certain immune cells, including macrophages and lymphocytes.[1,2] Stimulation of $P2X_7$ is proinflammatory, resulting in the release of inflammatory cytokines, such as interleukin (IL)-1 beta (β) and IL-18 from macrophages[2–5] and IL-2 from T cells,[5–7] as well as changes in the plasma membrane lipid distribution and cell death by necrosis or apoptosis.[2,3] $P2X_7$ has been reported to be involved in various nephropathy models, such as glomerular injury caused by diabetes and hypertension,[8] unilateral ureteral obstruction[9] and glomerulonephritis.[10] Dahl salt-sensitive (DS) rats fed on a high-sodium diet characteristically develop attenuated pressure natriuresis, hypertension and progressive renal injury, and this model has been widely used to study salt-sensitive hypertension.[11,12] In a previous study, we performed a genome-wide quantitative trait locus mapping analysis for blood pressure by using F2 rats derived from DS and Lewis (LEW) rats, and we identified a region on chromosome 12 near the *D12Arb6* marker that influenced blood pressure.[13] The *$P2X_7$* gene is also near the *D12Arb6* marker. A common genetic variation in the region of the *$P2X_7$* gene significantly affects blood pressure in a Caucasian population.[14] Although the pathophysiology of hypertension and renal diseases has been widely described, only a few studies have addressed the role of $P2X_7$ in the mechanism of these diseases. Therefore, the contribution of $P2X_7$ to salt-sensitive hypertension and renal injury has not been thoroughly investigated. In the present study, we examined the levels of renal $P2X_7$ in salt-sensitive hypertensive renal injury and the effects of $P2X_7$ receptor antagonists on the development of salt-sensitive hypertension and renal injury. Additionally, the role of the $P2X_7$ receptor in the macrophage activity of DS rats was also examined *in vitro*.

METHODS

Experimental animals

DS and LEW rats were obtained from SLC Japan (Shizuoka, Japan). These animals were kept in a pathogen-free environment, and experiments were performed according to the guidelines for the care and use of experimental animals of the National Cerebral and Cardiovascular Center and the National Institutes of Health. Adequate measures were taken to minimize pain and discomfort. Rats were housed in a temperature-controlled room with 12 h of light provided from 0700 to 1900 every day, and were fed normal or high-salt diets [0.5% (w/w) or 8% NaCl (w/w), respectively; Oriental Yeast, Tokyo, Japan], and provided with tap water ad libitum.

Assessment of $P2X_7$ expression in the rat kidney

DS and LEW rats were fed a normal or high-salt diet for 6 weeks from 4 weeks of age (N=6 for each group). At the conclusion of the study, the left kidneys of these rats were removed and renal weights were measured. RNA was isolated

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from the kidneys with Trizol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. $P2X_7$ mRNA expression levels were determined by real-time RT-PCR, by using a commercial kit (Rn00570451_m1; Applied Biosystems, Foster City, CA, USA), and normalized for the expression of GAPDH mRNA (4352338E; Applied Biosystems). These measurements were expressed as $\log_{10}[2^{35\text{-CT1}}/2^{25\text{-CT2}}]$, where $2^{35\text{-CT1}}$ and $2^{25\text{-CT2}}$ correspond to the expression levels of $P2X_7$ and GAPDH mRNA, respectively.

Western blot analysis was performed using the extracts from rat kidneys that had been frozen in liquid nitrogen and homogenized in Triton-based lysis buffer, as previously reported.[13] Equal amounts of protein (30 μg) were separated by SDS-PAGE (7.5% gels) and transferred to a PVDF membrane (Hybond-P; GE Healthcare, Buckinghamshire, UK). After blocking with 5% (w/v) skim milk powder, membranes were incubated with the primary rat $P2X_7$ antibody (APR-008; Alomone Labs, Jerusalem, Israel) at 1:1000 dilution, and the membrane-bound antibody was visualized with horseradish peroxidase-conjugated secondary antibody (NA934V; Amersham Biopharmacia, Buckinghamshire, UK), diluted 1:10 000, and an ECL advance western blotting detection kit (GE Healthcare). β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as a loading control.

Blockade of $P2X_7$ receptors in the DS rats

DS rats were fed a high-salt diet from 4 weeks of age. Blood pressure was measured weekly by a tail-cuff method (BP-98A; Softron, Tokyo, Japan). Rats were housed in metabolic cages for weekly 24-h urine collection. The $P2X_7$ antagonist,[10,15–18] brilliant blue G (BBG; Sigma, Dorset, UK), was administered as follows: Rats were treated with BBG for 4 weeks. BBG was diluted to $3\,\text{mg ml}^{-1}$ in calcium- and magnesium-free phosphate-buffered saline. DS rats (N=7) were administered BBG ($50\,\text{mg kg}^{-1}$) i.p. every 24 h; control DS rats (N=7) were administered only vehicle solutions. Urinary protein level was measured using a BCA protein assay kit (Thermo Scientific, Rockford, IL, USA). Creatinine clearance (C_{Cr}) on week 4 of the administration was measured, which was calculated using the formula: $C_{Cr}=(U_{Cr}\times V)/P_{Cr}$, where U_{Cr} is the concentration of urinary creatinine (mg dl^{-1}), P_{Cr} is the concentration of plasma creatinine (mg dl^{-1}), and V is the urine flow rate (ml min^{-1}). Creatinine levels were measured using a QuantiChrom creatinine assay kit (DIUR-500; Bioassay Systems, Hayward, CA, USA).

Another $P2X_7$ antagonist, A-438079 (Tocris Bioscience, Bristol, UK), was also used in this study. DS rats fed with the high-salt diet, were treated with A-438079 for 7 days. A-438079 was diluted to $3\,\text{mg ml}^{-1}$ in isotonic saline solution. DS rats (N=8) were administered A-438079 ($100\,\text{mg kg}^{-1}$) i.p. every 24 h; control DS rats (N=11) were administered only the vehicle solution. Albumin levels were measured using an enzyme-linked immunosorbent assay kit (Shibayagi, Gunma, Japan).

Histological examination

Kidney tissue was fixed in 10% formaldehyde and embedded in paraffin. Sections were prepared and stained with hematoxylin and eosin and Masson's trichrome. Tubulointerstitial injury in the renal cortex was assessed according to the percentage area of fibrosis, estimated using the Masson's trichrome-stained sections. For each kidney, 10 microscopic fields (×400 magnification) were randomly chosen, using a fluorescence microscope (BZ-9000; Keyence, Osaka, Japan), and the area of renal fibrosis was measured and analyzed using analysis software (BZ image analyzer II; Keyence).

Immunohistochemistry

Paraffin-embedded sections were deparaffinized. After blocking with 5% goat serum, kidney sections were incubated with antibody against $P2X_7$ (APR-004; Alomone Labs, Jerusalem, Israel), CD68 (Abcam, Cambridge, UK), or CD3 (DAKO, Glostrup, Denmark) as primary antibodies overnight at 4 °C, followed by Simplestain MAX-PO (rat) as a secondary antibody (Nichirei, Tokyo, Japan). Antibody binding was visualized using 3,3-diaminobenzidene (Dojindo Laboratories, Kumamoto, Japan) and counterstained with hematoxylin. The images were captured at high-power magnification (×400) with 10 fields for each kidney, using a fluorescence microscope (BZ-9000; Keyence). The number of macrophages and T cells were counted and the results were expressed as the number of positive cells per mm^2 of kidney tissue. To determine the association between $P2X_7$ receptors and macrophages, the expression sites of $P2X_7$ and CD68 in consecutive sections were determined.

Assessment of IL-1β secretion by macrophages

Peritoneal macrophages were collected as previously described.[19,20] Briefly, rats (DS and LEW, N=13, fed a normal diet) were injected i.p. with sterile 3% thioglycolate medium, and 4 days later, the peritoneal exudate corpuscles were collected by lavage with phosphate-buffered saline. Cell suspensions were seeded in six-well cell culture plates (2×10^6 cells per well) in RPMI-1640 medium containing 10% fetal bovine serum and 2 mM of L-glutamine (>2 wells for each rat). After 4 h of incubation at 37°C (5% CO_2 atmosphere), non-adherent cells were removed using fresh RPMI-1640 medium. These cells were then incubated for 48 h at 37 °C and primed with $10\,\text{ng ml}^{-1}$ LPS

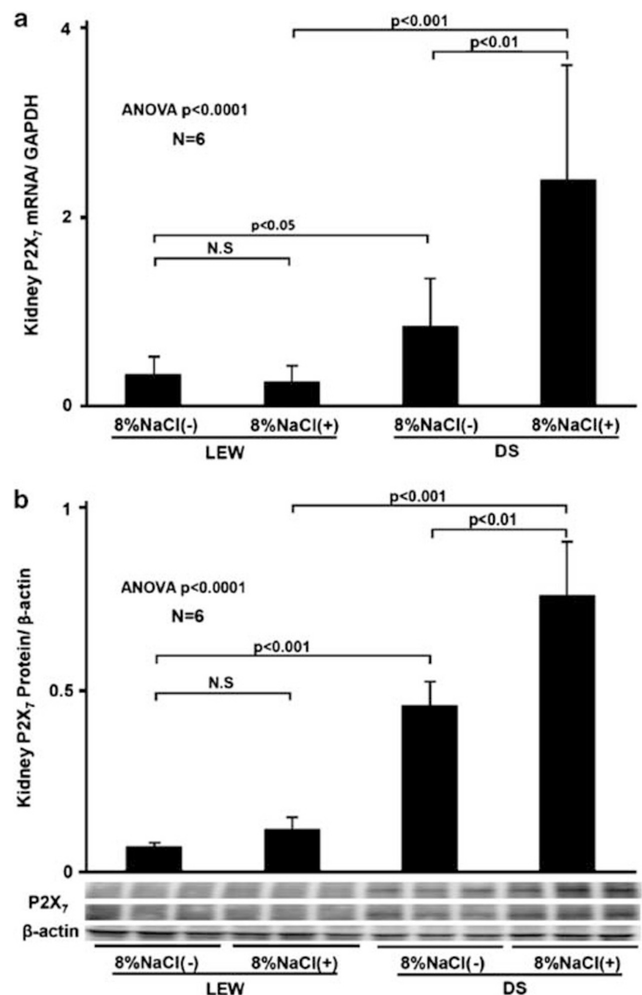

Figure 1 $P2X_7$ expression in the kidneys of DS and LEW rats. (**a**) $P2X_7$ mRNA levels in the kidneys of the DS and LEW rats. Real-time RT-PCR analyses were performed using GAPDH mRNA levels as an internal control. The figure shows a summary of the data analyzed by analysis of variance (ANOVA) (N=6 for each group). Multiple regression analysis indicated that $P2X_7$ mRNA levels in the kidneys were significantly influenced by the difference in the strains (DS or LEW) and by the amount of salt in the diet (high- or normal-salt diet). $P2X_7$ mRNA levels in the kidneys of the DS rats were significantly higher than those in the kidneys of the LEW rats (DS *vs.* LEW, $P<0.0001$, by ANOVA). (**b**) $P2X_7$ protein expression in the kidneys of the DS and LEW rats. Western blot analysis indicated that $P2X_7$ protein levels in the kidneys of the DS rats were significantly higher than those in the kidneys of the LEW rats (N=6 for each group) (DS *vs.* LEW, $P<0.0001$, by ANOVA).

(Sigma, Poole, UK) for 12 h before RNA was extracted to measure the expression levels of $P2X_7$ mRNA. For the assessment of IL-1β release, the remaining cells were stimulated for 6 h with 100 μM of the $P2X_7$ agonist, BzATP,[17] which is a mixture of 2′- and 3′-O-(4-benzoyl-benzoyl) adenosine 5′-triphosphate (Sigma, St Louis, MO, USA). The medium was then transferred to a new tube and centrifuged at 14 000 r.p.m. to remove any non-adherent cells, and the clear supernatants were stored at −80 °C until use. IL-1β was measured in duplicate with an enzyme-linked immunosorbent assay kit (IBL, Gunma, Japan).

Statistical analysis

Statistical analysis of experimental data was performed with the JMP statistical software package (SAS Institute, Cary, NC, USA), and the results are expressed as mean ± s.d. Analysis of variance or multivariate analysis of variance was used to estimate the statistical difference among groups, and significant differences were further analyzed by Student's *t*-test.

RESULTS

$P2X_7$ expression is higher in the kidneys of DS rats

Transcriptome analysis indicated that $P2X_7$ mRNA was expressed more abundantly in the kidneys of the DS than in the kidneys of the LEW rats with a normal diet (Figure 1a). Multiple regression analysis indicated that $P2X_7$ mRNA levels were higher in the DS than in the LEW rats fed a diet containing 8% NaCl ($P<0.001$), and it also indicated that feeding a high-salt diet influenced $P2X_7$ mRNA levels in the DS rat kidney ($P<0.01$), but not in the LEW rat kidney. Western blot analysis showed corresponding results with $P2X_7$ protein expression levels (Figure 1b).

Blockade of $P2X_7$ in the DS rats

One of the major histological findings in the kidneys of DS rats was the infiltration of immune cells (macrophages and T cells) into the renal interstitium.[21,22] It is likely that a higher responsiveness of the $P2X_7$ pathway in the DS rats may aggravate inflammation in the kidney that has been damaged by hypertension. High-salt-containing diets in DS rats induced increased blood pressure, urinary protein excretion, renal interstitial fibrosis, macrophage infiltration and T-cell infiltration. Concomitant administration of BBG, an antagonist of the rat $P2X_7$ receptor, significantly attenuated the rise in blood pressure on and after week 2 of the administration (Figure 2a), and renal weight (Figure 2b) and urinary protein excretion on and after week 3 of the administration (Figure 2c). Creatinine clearance also significantly improved on week 4 of the administration (Figure 2d). Renal

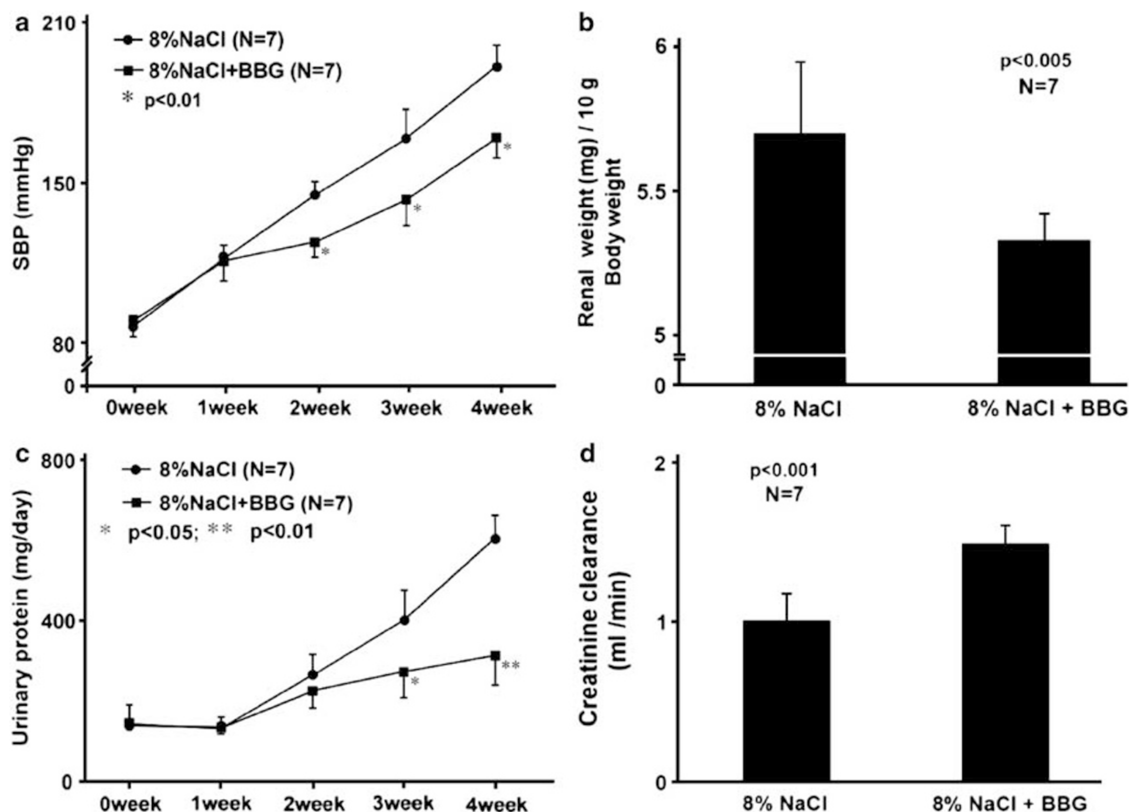

Figure 2 Blockade of $P2X_7$ in the DS rats with BBG. (**a**) Effects on blood pressure. In all 4 weeks of the 8% NaCl diet significantly increased blood pressure in the DS rats (N=7 for both groups). Multivariate analysis of variance (MANOVA) indicated that BBG treatment significantly attenuated this rise in blood pressure ($P<0.0001$). *$P<0.01$, significantly different from the values obtained in the 8% NaCl diet group (Student's *t*-test). (**b**) Effects on renal weight. Kidneys were harvested at 4 weeks with or without BBG treatment (N=7 for both groups), and renal weight was represented as a relative weight (renal organ weight per 10 g of body weight). Renal weight in the DS rats without BBG treatment was significantly higher than in those with BBG treatment ($P<0.005$, Student's *t*-test). (**c**) Effects on urinary protein excretion. A total of 4 weeks of the 8% NaCl diet significantly increased urinary protein excretion in the DS rats (N=7 for both groups). MANOVA indicated that BBG treatment significantly attenuated this increase in urinary protein excretion (P=0.0006). *$P<0.05$, **$P<0.01$, significantly different from the values obtained in the 8% NaCl diet group (Student's *t*-test). (**d**) Effects on creatinine clearance. Creatinine clearance (C_{Cr}) at 4 weeks was measured, which was calculated using the formula: $C_{Cr}=(U_{Cr}\times V)/P_{Cr}$, where U_{Cr} is the concentration of urinary creatinine (mg dl^{-1}), P_{Cr} is the concentration of plasma creatinine (mg dl^{-1}), and V is the urine flow rate (ml min^{-1}). Creatinine clearance significantly improved by 4 weeks of BBG treatment ($P<0.001$, Student's *t*-test).

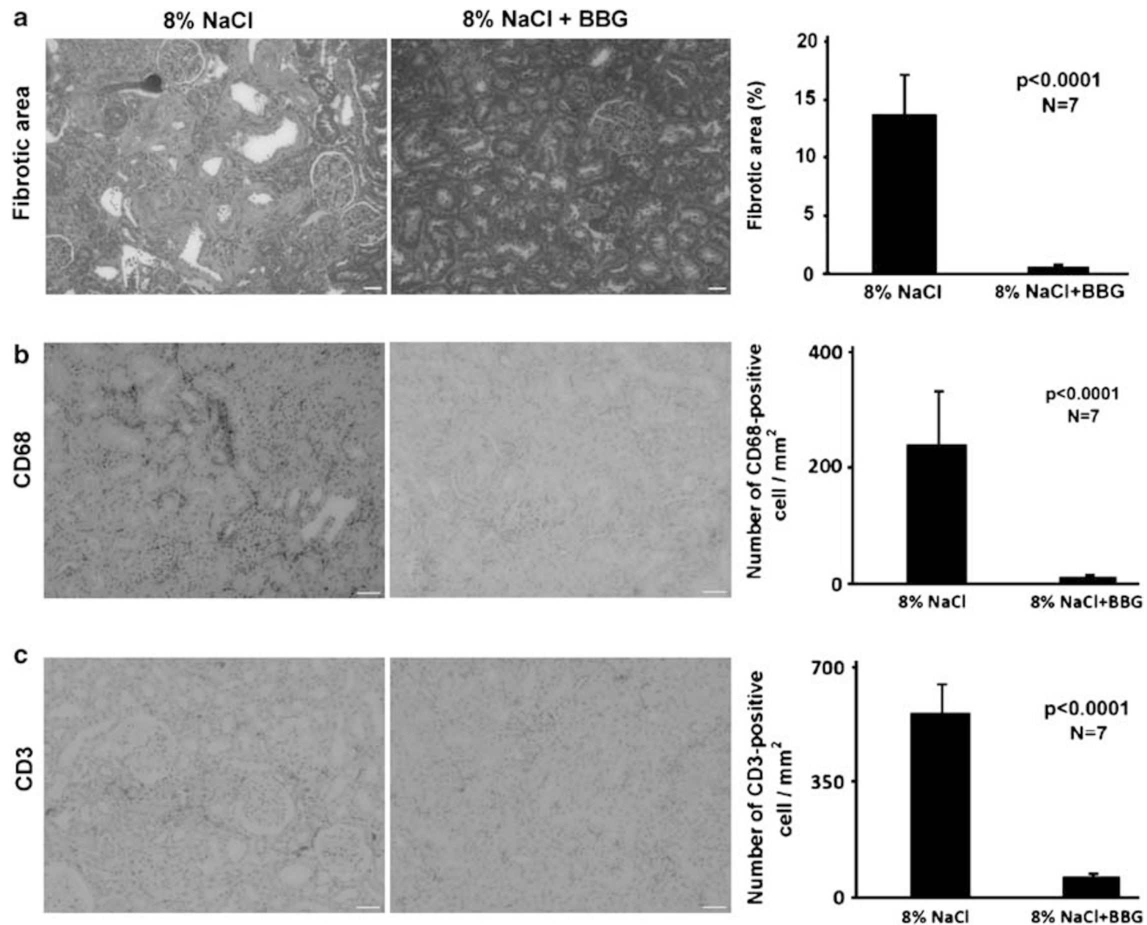

Figure 3 Effect on interstitial fibrosis and immune cell infiltration of the kidneys of DS rats with BBG. (**a**) Effects on interstitial fibrosis. In all 4 weeks of the 8% NaCl diet caused tubular dilatation, interstitial fibrosis and glomerular sclerosis (N=7 for both groups). BBG treatment significantly attenuated these pathological changes. The percentage of fibrotic area as assessed by blue staining on Masson's trichrome-stained sections was markedly reduced by BBG treatment. (**b**) Effects on macrophage infiltration. A total of 4 weeks of the 8% NaCl diet significantly increased macrophage infiltration (N=7 for both groups). On immunohistochemical examination, macrophages were identified by positive staining for anti-CD68 antibody. BBG treatment markedly attenuated macrophage infiltration. (**c**) Effects on T-cell infiltration. In all 4 weeks of the 8% NaCl diet strongly increased T-cell infiltration (N=7 for both groups). On immunohistochemical examination, T cells were identified by positive staining for CD3 antibody. BBG treatment markedly attenuated T-cell infiltration. Scale bars=50 μm.

interstitial fibrosis (Figure 3a), macrophage infiltration (Figure 3b) and T-cell infiltration (Figure 3c) were attenuated by BBG treatment.

Moreover, another $P2X_7$ antagonist, A-438079, also attenuated 24-h urinary albumin excretion, on and after day 2 of the administration (Figure 4), and renal macrophage infiltration (Figure 5).

$P2X_7$ expression is higher in macrophages of DS rats

$P2X_7$ mRNA was expressed more abundantly in peritoneal macrophages derived from the DS rats than in those from the LEW rats (Figure 6a). LPS stimulation is known to promote the accumulation of IL-1β in macrophages.[2,23,24] In response to the $P2X_7$ agonist, BzATP (100 μM), LPS-primed macrophages from the DS rats released more IL-1β than those from the LEW rats (Figure 6b).

Co-localization of $P2X_7$ and macrophages in the DS rat kidney

To determine the association between $P2X_7$ and macrophages in the kidneys of the DS rats, expression site studies in consecutive sections were carried out; $P2X_7$ receptor immunoreactivity co-localized with positive macrophages (Figure 7).

DISCUSSION

$P2X_7$ is recognized as one of the key factors in the modulation of inflammatory processes.[2,3,25] Indeed, $P2X_7$ has recently been reported to be involved in various inflammatory processes of different pathological conditions, such as cerebral infarction[26] or ischemia,[27,28] lung inflammation and emphysema,[29] allergic airway inflammation,[30] unilateral urethral obstructive nephropathy[9] and accelerated nephrotoxic nephritis.[10] Several recent studies have provided convincing evidence that the renal proinflammmatory response has an important role in mediating salt-sensitive hypertension.[31,32]

In a previous study, quantitative trait locus analysis identified the *$P2X_7$* gene as a candidate gene for hypertension, by using F2 rats derived from DS and LEW rats.[13] In the present study, expression levels of $P2X_7$ mRNA and protein in the kidneys of DS were much higher than those in the kidneys of LEW with normal diets. These high levels of $P2X_7$ mRNA and protein may result in renal inflammation in DS, leading to salt-sensitive hypertension and renal injury. After feeding of a high-salt diet, the $P2X_7$ mRNA and protein levels were substantially higher in the DS kidney. In contrast, in the LEW

kidney, $P2X_7$ mRNA and protein levels were essentially unchanged. These results suggested that $P2X_7$ levels had a relationship with salt-sensitive hypertension and renal injury.

To test this hypothesis, we investigated the effects of two $P2X_7$ receptor antagonists on salt-sensitive hypertension and renal injury. BBG is a potent antagonist of the rat $P2X_7$ receptor, and it is associated with low toxicity[33] and high selectivity.[15] The only notable side effect is a temporary blue tint of the skin. Another $P2X_7$ receptor antagonist, A-438079, selectively blocks the $P2X_7$ receptor,[34] and shows a high degree of selectivity for $P2X_7$ in humans, rats and mice.[10,17,18] Within 4 weeks of treatment, BBG markedly attenuated salt-sensitive hypertension, urinary protein excretion, interstitial fibrosis and macrophage and T-cell infiltration in DS rats that received the high-salt diet. A-438079 treatment for 7 days also significantly attenuated 24-h urinary albumin excretion and macrophage infiltration in the DS rats fed the high-salt diet. Thus, these data strongly support the hypothesis that $P2X_7$ is involved in hypertensive renal injury. The $P2X_7$ receptor antagonists can lower blood pressure and provide renal protection in salt-sensitive hypertension, confirming that $P2X_7$ is an important therapeutic target for renal diseases, including hypertension.

Although the mechanism by which $P2X_7$ affects salt-sensitive hypertension and renal injury in DS rats is still unclear, one hypothesis is increased activation of macrophages or T cells in DS rats. The proinflammatory $P2X_7$ receptor is expressed mainly in immune

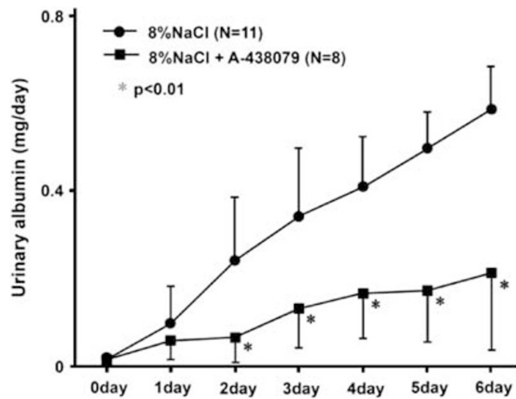

Figure 4 Effects on urinary albumin excretion of DS rats with A-438079. The 24-h urinary albumin excretion increased in response to the 8% NaCl diet (N=11 for the control group and N=8 for the A-438079 group). MANOVA indicated that A-438079 treatment significantly attenuated this increase in urinary albumin excretion (P<0.0001). *P<0.01, significantly different from the values obtained in the 8% NaCl diet group (Student's *t*-test).

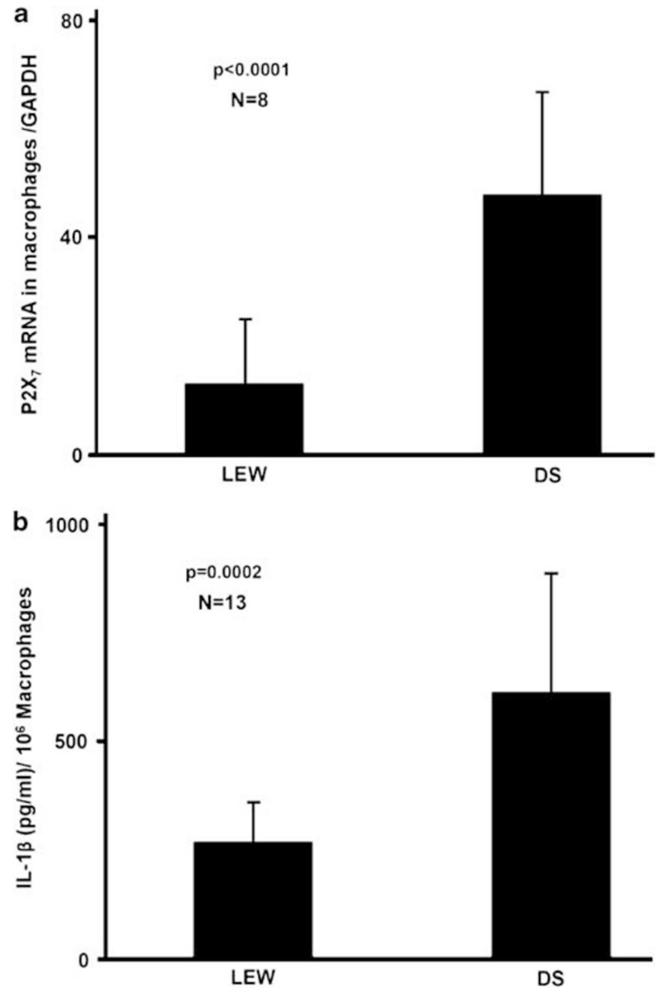

Figure 6 $P2X_7$ in peritoneal macrophages from the DS and LEW rats without salt loading. (**a**) $P2X_7$ mRNA levels in the peritoneal macrophages from the DS and LEW rats. Real-time RT-PCR analyses were performed using GAPDH mRNA levels as an internal control. The figure shows a summary of the data analyzed by ANOVA (N=8 for each group). $P2X_7$ mRNA levels in the macrophages from the DS rats were significantly higher than those in the macrophages from the LEW rats (DS *vs.* LEW, P<0.0001). (**b**) IL-1β release from the LPS-primed macrophages. Peritoneal macrophages were isolated from the DS rats (N=13) and LEW rats (N=13). LPS-primed macrophages were subjected to BzATP stimulation (100 μM, for 6 h at 37 °C) for the assessment of IL-1β release. Significantly higher release of IL-1β was observed from the macrophages of the DS rats compared with that from the macrophages of the LEW rats (P=0.0002).

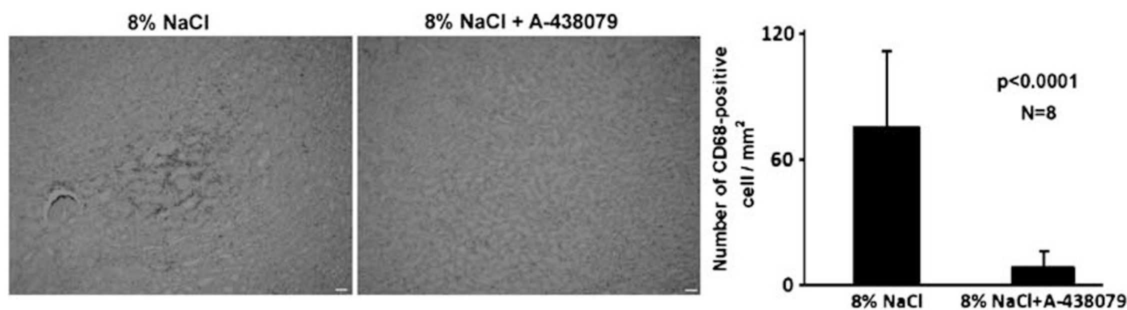

Figure 5 Effects on macrophage infiltration of the kidneys of DS rats with A-438079. A total of 7 days of feeding with the high-salt diet strongly increased macrophage infiltration in the renal interstitium of the DS rats. A-438079 treatment for 7 days markedly attenuated macrophage infiltration. On immunohistochemical analysis, macrophages in the kidney were identified by anti-CD68-positive staining. Scale bars=50 μm.

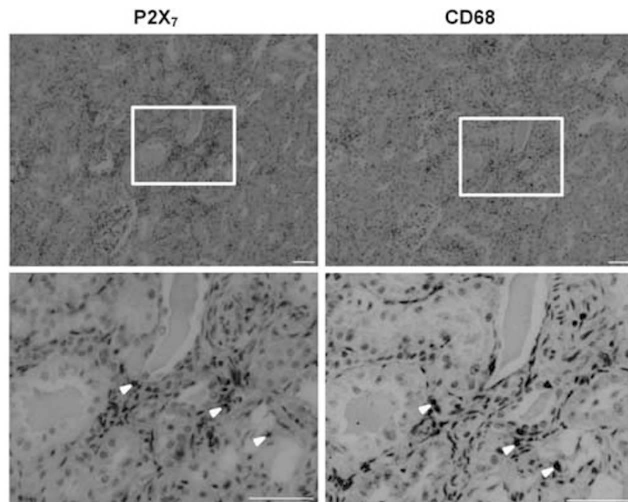

Figure 7 Co-localization of $P2X_7$ and CD68 (macrophage marker) in renal consecutive sections. On immunohistochemical examination, $P2X_7$ and macrophages were identified by positive staining with anti-$P2X_7$ and anti-CD68 antibodies, respectively. It is shown that macrophages are co-expressed with $P2X_7$ (arrowheads). Lower panels are magnified photographs of upper ones (rectangular area). Scale bars=50 μm.

cells.[2,23] In addition, it has been reported that inflammatory responses in which $P2X_7$ is involved proceed via release of IL-1β.[2,35–37] Therefore, $P2X_7$ mRNA levels in macrophages from DS and LEW rats were measured, and macrophage $P2X_7$ expression was higher in DS than in LEW rats. The experiment on the activation of macrophages from DS and LEW rats revealed that LPS-primed macrophages from the DS rats released more IL-1β in response to BzATP than the macrophages from the LEW rats. These results indicate that renal inflammatory responses are probably exacerbated by a higher responsiveness of the $P2X_7$ pathway in immune cells (macrophages and T cells). Furthermore, co-localization of $P2X_7$ and macrophages (CD68-positive cells) in consecutive sections of the kidney also supported this hypothesis.

Our results provide support for the following hypothesis. High-salt intake evokes an initial elevation of blood pressure, which is likely to cause slight renal injury and subsequent release of cytokines from inflammatory cells in the kidney. This mild inflammatory response causes ATP release. This extracellular ATP may be diffuse and may activate the $P2X_7$ receptors of macrophages and T cells, and IL-1β and IL-2 are released from these cells.[1] Then, IL-1β and IL-2 possibly increase the cytokine production by the macrophages and T cells, which further enhances inflammation.[2,3] This severe inflammation exacerbates the renal injury and impairs function, and blood pressure is elevated further. Thus, $P2X_7$ may have contributed to a particularly severe cycle of hypertension and renal injury in the DS rats through its higher expression in immune cells.

Our results indicate that the differences in expression levels of macrophage $P2X_7$ between DS and LEW rats contribute to the progression of renal injury, but this was not directly proved. However, $P2X_7$ antagonism markedly attenuated salt-sensitive hypertension and renal injury, and macrophages with high $P2X_7$ expression release more IL-1β. Therefore, these results are regarded as indirect evidence of our conclusion.

The pathogenesis of salt-sensitive hypertension and renal injury is very complicated in DS rats. Furlan-Freguia *et al.*[38] recently reported that the $P2X_7$ receptor was involved in thrombosis. As thrombosis at renal sites of inflammation may exacerbate renal injury, this is also a possible cause of salt-sensitive hypertension and renal injury in DS rats. $P2X_7$ may also play significant roles in cell types other than immune cells, some of which might also be involved in blood pressure regulation. Further studies are needed to investigate these speculations and whether the high expression level of $P2X_7$ in DS rats is due to genetic differences or a secondary response.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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