

Figure 3 Testosterone concentrations in testicular tissue of SHRSP given diet containing soybean oil (Control diet), Healthy Resetta[®], Healthy Choleste[®], and Kenko Sarara[®] for 4 weeks.

HR, Healthy Resetta[®] ; HC, Healthy Choleste[®] ; KS, Kenko Sarara[®]

Values are means \pm SEM of 6 animals.

No differences in mean values were found between control group and any other group (One-way ANOVA followed by Dunnett's test).

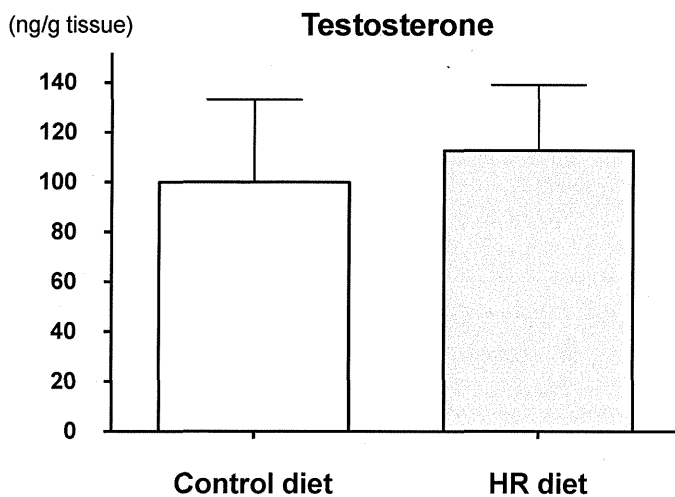
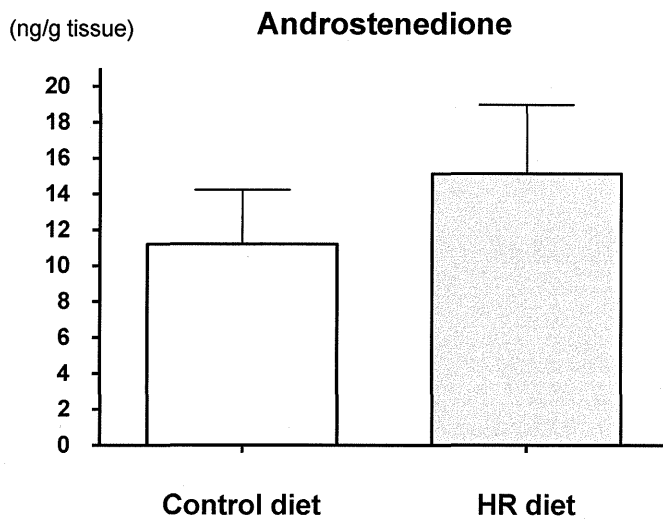
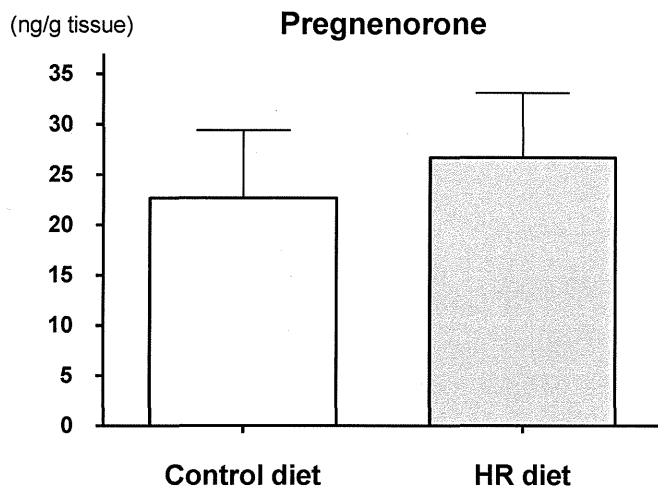


Figure 4

Pregnenorone, androstenedione and testosterone concentrations in testicular tissue of SHRSP given diet containing soybean oil (Control diet) or Healthy Resetta[®], for 4 weeks.

HR, Healthy Resetta[®].

Values are means \pm SEM of 6 animals.

No differences were found in mean values of each steroid hormone between control group and HR group (Student's t-test).

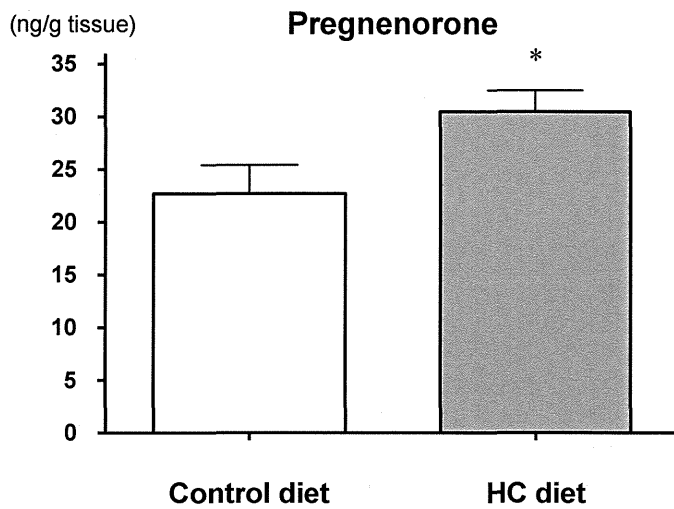


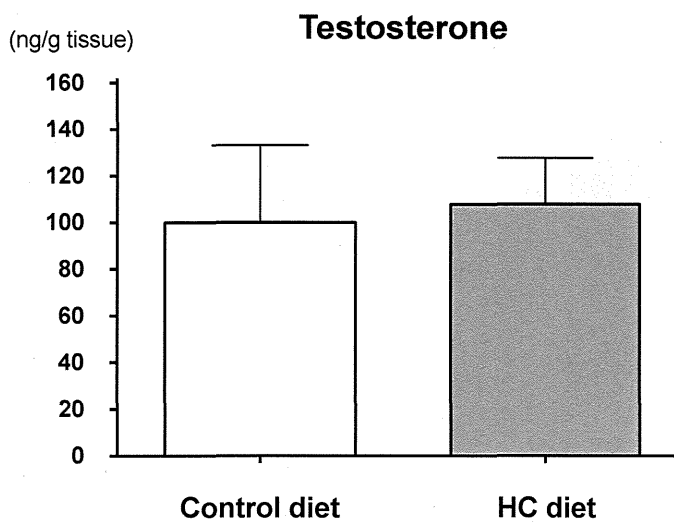
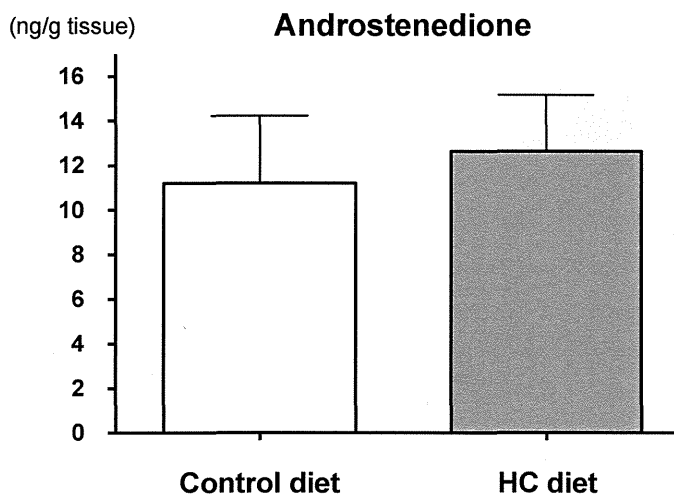
Figure 5

Pregnenorone, androstenedione and testosterone concentrations in testicular tissue of SHRSP given diet containing soybean oil (Control diet) or Healthy Choleste® for 4 weeks.

HC, Healthy Choleste®.

Values are means ± SEM of 6 animals.

* $p < 0.05$, significantly different (Student's t-test).



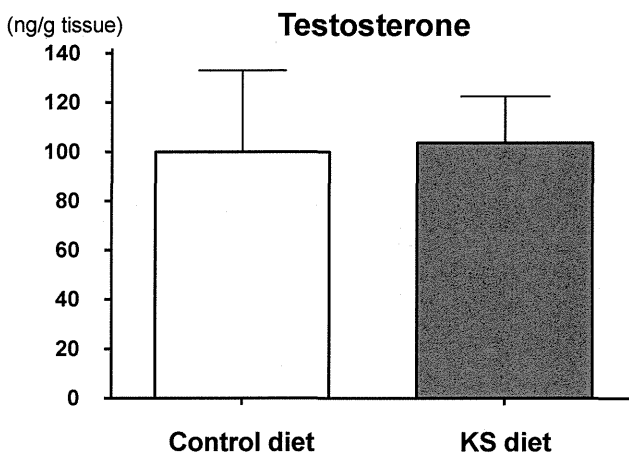
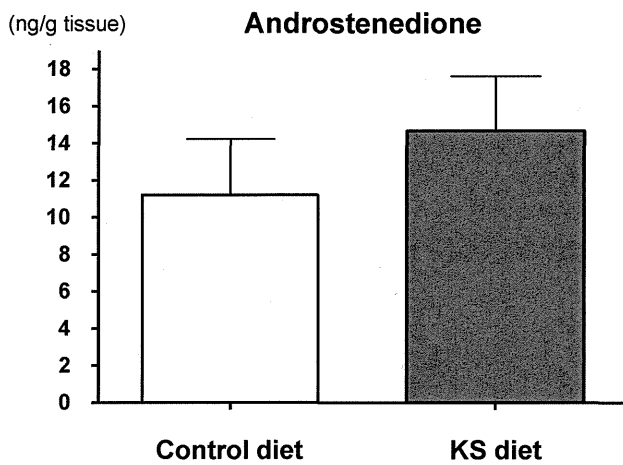
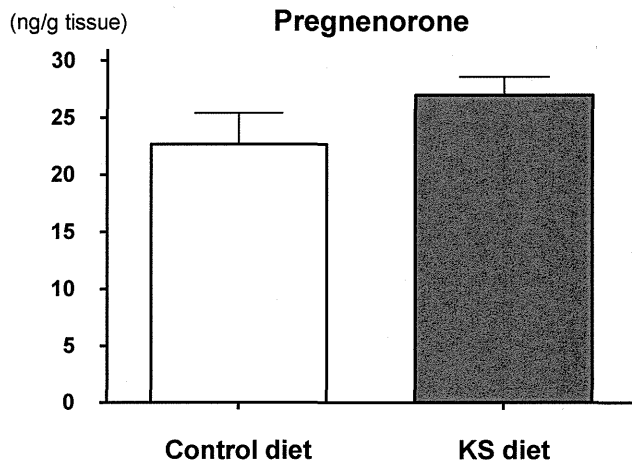


Figure 6

Pregnenorone, androstenedione and testosterone concentrations in testicular tissue of SHRSP given diet containing soybean oil (Control diet) or Kenko Sarara® for 4 weeks.

KS, Kenko Sarara®

Values are means \pm SEM of 6 animals.

No differences were found in mean values of each steroid hormone between control group and HR group (Student's t-test).

Ⅲ. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

雑誌

発表者名	論文タイトル名	発表誌名	巻号	ページ	出版年
Xu Ji, Yukiko Naito, Huachun Weng, Kosuke Endo, Xiao Ma, Naoharu Iwai	P2X7 deficiency attenuates hyper- tension and renal injury in deoxy- corticosterone acetate-salt hy- pertension	Am. J. Physiol. Renal Physiol.	303	F1207 - F1215	2012

IV. 研究成果の刊行物・別刷

P2X₇ deficiency attenuates hypertension and renal injury in deoxycorticosterone acetate-salt hypertension

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Ji X, Naito Y, Weng H, Endo K, Ma X, Iwai N. P2X₇ deficiency attenuates hypertension and renal injury in deoxycorticosterone acetate-salt hypertension. *Am J Physiol Renal Physiol* 303: F1207–F1215, 2012. First published August 1, 2012; doi:10.1152/ajprenal.00051.2012.—The P2X₇ receptor is a ligand-gated ion channel, and genetic variations in the P2X₇ gene significantly affect blood pressure. P2X₇ receptor expression is associated with renal injury and inflammatory diseases. Uninephrectomized wild-type (WT) and P2X₇-deficient (P2X₇ KO) mice were subcutaneously implanted with deoxycorticosterone acetate (DOCA) pellets and fed an 8% salt diet for 18 days. Their blood pressure was assessed by a telemetry system. The mice were placed in metabolic cages, and urine was collected for 24 h to assess renal function. After 18 days of DOCA-salt treatment, P2X₇ mRNA and protein expression increased in WT mice. Blood pressure in P2X₇ KO mice was less than that of WT mice (mean systolic blood pressure 133 ± 3 vs. 150 ± 2 mmHg). On day 18, urinary albumin excretion was lower in P2X₇ KO mice than in WT mice (0.11 ± 0.07 vs. 0.28 ± 0.07 mg/day). Creatinine clearance was higher in P2X₇ KO mice than in WT mice (551.53 ± 65.23 vs. 390.85 ± 32.81 μl·min⁻¹·g renal weight⁻¹). Moreover, renal interstitial fibrosis and infiltration of immune cells (macrophages, T cells, B cells, and leukocytes) were markedly attenuated in P2X₇ KO mice compared with WT mice. The levels of IL-1β, released by macrophages, in P2X₇ KO mice had decreased dramatically compared with that in WT mice. These results strongly suggest that the P2X₇ receptor plays a key role in the development of hypertension and renal disease via increased inflammation, indicating its potential as a novel therapeutic target.

P2X₇; hypertension; renal injury; inflammation; immune cell

THE P2X₇ RECEPTOR is an ATP-gated cation channel (27), primarily expressed by various immune cells including macrophages (17, 20, 23) and lymphocytes (32, 33). Stimulation of this receptor leads to the release of IL-1β from macrophages (17, 20) and IL-2 from T cells (32, 33). The activation of macrophages and T cells in P2X₇-deficient (P2X₇ KO) mice is lower than that in wild-type (WT) mice (3, 28). The P2X₇ receptor plays a key role in inflammatory reactions, which are involved in various diseases (1, 7). Moreover, the P2X₇ receptor is involved in diverse renal disease models such as glomerular injury due to diabetes and hypertension (30), unilateral ureteral obstruction (10), renal injury due to salt-sensitive hypertension (13), and glomerulonephritis (29). Previously, we performed a genomewide quantitative trait locus analysis for rat blood pressure and found that the region around the *D12Arb6* marker (located near the P2X₇ gene) is involved in the regulation of blood pressure (12). It is also reported that P2X₇ genetic variation significantly affects blood pressure in a Caucasian population (19). Although the pathophysiology of

hypertension and renal diseases is well studied (14, 31), few studies address the role of the P2X₇ receptor in the progression of hypertension and renal diseases. Therefore, the relevance of the P2X₇ receptor in hypertension and renal injury remains to be elucidated. Elucidating the factors responsible for deoxycorticosterone acetate (DOCA)-salt hypertension will enhance our understanding of the mechanisms of hypertension resulting from hypervolemia, hyperaldosteronism, and high salt intake. In the present study, we investigated the role of the P2X₇ receptor in the development of DOCA-salt hypertension and renal injury in WT and P2X₇ KO mice. We examined the possible effects of the P2X₇ receptor on blood pressure, renal function (i.e., urinary albumin and creatinine clearance), and renal injury (i.e., area of fibrosis). Furthermore, we investigated the possible effects of the P2X₇ receptor on infiltration of immune cells (i.e., macrophages, T cells, B cells, and leukocytes) in the kidneys and IL-1β release from macrophages. The possible effects of the P2X₇ receptor on cyclooxygenase-2 (COX-2) expression and total serum antioxidant activity were also examined in this study.

MATERIALS AND METHODS

Experimental animals. Male WT (C57BL/6J) mice were obtained from SLC Japan (Shizuoka, Japan), and male P2X₇ KO mice (26) were obtained from Jackson Laboratory (Bar Harbor, ME). P2X₇ KO mice were congenic with the C57BL/6J genetic background. The animals used in the experiments were 10–12 wk old. The mice were housed in a temperature-controlled pathogen-free room with light from 0700 to 1900 (daytime) and had free access to food and water. The experimental protocols were approved by the National Cerebral and Cardiovascular Center Committee for Laboratory Animals, and the care and use of the animals were performed in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Measurement of blood pressure and expression of target mRNA in DOCA-salt hypertension mice. Before the DOCA-salt treatment, telemetry system transmitters (Data Sciences International, St. Paul, MN) were implanted (6). Both WT and P2X₇ KO mice were anesthetized with pentobarbital (25 mg/kg), and a transmitter catheter was inserted into the left carotid artery, with the transmitter body placed subcutaneously in the lower right side of the abdomen. Simultaneously, the left kidney was removed as baseline sample and cut longitudinally. One half of each kidney was frozen in liquid nitrogen, while the other half was fixed in 4% formalin.

Ten days after radiotelemetry implantation, mice were reanesthetized with pentobarbital (25 mg/kg), and a DOCA pellet (0.12 mg·g body wt⁻¹·day⁻¹; Innovative Research of America, Sarasota, FL) was implanted subcutaneously in the neck region. Mice were fed an 8% NaCl diet, and the treatment with DOCA-salt was continued for 18 days.

Heart rate (HR), systolic blood pressure (SBP), diastolic blood pressure (DBP), and pulse pressure (PP) were measured throughout the DOCA-salt treatment period with the telemetry system according to the manufacturer's instructions. In addition, on day 18, mice were

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housed for 24 h in metabolic cages to collect urine for measurement of albumin levels and creatinine clearance (C_{Cr}). Urine was stored at -80°C until renal function evaluation.

After the DOCA-salt treatment, mice were anesthetized with pentobarbital (25 mg/kg). Venous blood was collected from the vena cava. Plasma (EDTA as anticoagulant) and serum were isolated by centrifugation and stored at -80°C until measurement. The kidneys from each mouse were removed, weighed, and cut longitudinally. One half of each kidney was frozen in liquid nitrogen and used exclusively for the isolation of total RNA, while the other half was fixed in 4% formalin and used for histological analyses. Renal weight was expressed as renal organ weight per 10 g of body weight. RNA was isolated from the mouse kidneys with TRIzol reagent (Invitrogen, Carlsbad, CA). P2X₇ mRNA expression was measured by real-time RT-PCR analysis with commercial kits (Applied Biosystems, Foster City, CA); the levels were normalized to the expression of β -actin mRNA. Measurements are expressed as $\log_{10}[2^{35 - CT1}/2^{25 - CT2}]$, where $2^{35 - CT1}$ and $2^{25 - CT2}$ correspond to the expression levels of the target and β -actin mRNA, respectively.

Renal function assessment. A series of experiments were further performed to test the renal function of the P2X₇ KO mice with the same DOCA-salt diet treatment described above. Urine was collected for 24 h from mice in metabolic cages on days 0, 2, 5, 8, and 18 to assess renal function.

Glomerular barrier function was determined by assessing urinary albumin excretion. The urinary albumin concentration was determined with an albumin enzyme-linked immunosorbent assay kit (Exocell; Shibayagi, Gunma, Japan).

Renal filtration function was evaluated by measuring C_{Cr} on day 18, which was calculated with the following formula: $C_{Cr} = (U_{Cr} \times V)/P_{Cr}/KW$, where U_{Cr} is the concentration of urinary creatinine (mg/dl), P_{Cr} is the concentration of plasma creatinine (mg/dl), V is the urine flow rate ($\mu\text{l}/\text{min}$), and KW is the weight of the kidney (g). Creatinine levels were measured with a QuantiChrom creatinine assay kit (DIUR-500; Bioassay Systems, Hayward, CA). Urinary sodium concentration was measured with a compact salt meter (C-121; Horiba, Kyoto, Japan). Urine samples of mice in the telemetry examination were also used to measure urinary volume, albumin level, and C_{Cr} on day 18.

Histological examination. Tubulointerstitial injury in the renal cortex was measured as the percentage of the area of fibrosis and estimated by Sirius red staining. The formaldehyde-fixed kidneys were embedded in paraffin before sections were prepared and stained with Sirius red. For each kidney, 10 microscopic fields ($\times 400$ magnification) were chosen randomly with a fluorescence microscope (BZ-9000; Keyence, Osaka, Japan), the area of renal fibrosis was measured and analyzed with analysis software (BZ image analyzer II; Keyence), and the mean value was calculated for each kidney.

Immunohistochemistry. Immunohistochemistry was used to detect P2X₇, macrophages, T cells, B cells, leukocytes, and podocytes. Paraffin sections were deparaffinized with xylene in a graded-alcohol series. After antigen retrieval, endogenous peroxidase activity was blocked by incubation in 3.3% H_2O_2 at room temperature for 30 min, followed by incubation in 5% normal goat serum for 1 h to block nonspecific binding. After thorough washing with phosphate-buffered saline (PBS), the sections were incubated overnight at 4°C with primary antibodies against P2X₇ (Alomone Labs, Jerusalem, Israel), F4/80 (Santa Cruz Biotechnology, Santa Cruz, CA), CD3 (DAKO, Glostrup, Denmark), CD20 (eBioscience, San Diego, CA), CD45 (BD Pharmingen, San Diego, CA), synaptopodin (Santa Cruz Biotechnology), and COX-2 (Cayman Chemical, Ann Arbor, MI). The Simplestain MAX-PO (mouse) kit (Nichirei, Tokyo, Japan) was used as a secondary antibody and incubated for 30 min at room temperature. Antibody binding was visualized with 3,3'-diaminobenzidine (DAB; Dojindo Laboratories, Kumamoto, Japan), and the nuclei were stained with hematoxylin. Images were captured at high-power magnification ($\times 400$) in 10 fields for each kidney with a fluorescence microscope

(BZ-9000, Keyence). The numbers of positive cells were counted, and the results were expressed as the number of positive cells per square millimeter of kidney tissue. To determine the relationship between P2X₇ receptors and macrophages in WT mice after DOCA-salt treatment, the expression sites of P2X₇ and F4/80 in consecutive sections were observed. In addition, to determine the relationship between P2X₇ receptors and podocytes in WT mice after DOCA-salt treatment, colocalization studies on P2X₇ and synaptopodin (a podocyte marker) in consecutive sections were performed.

Assessment of IL-1 β secretion by macrophages. Peritoneal macrophages were collected as described previously (3, 13). Briefly, mice

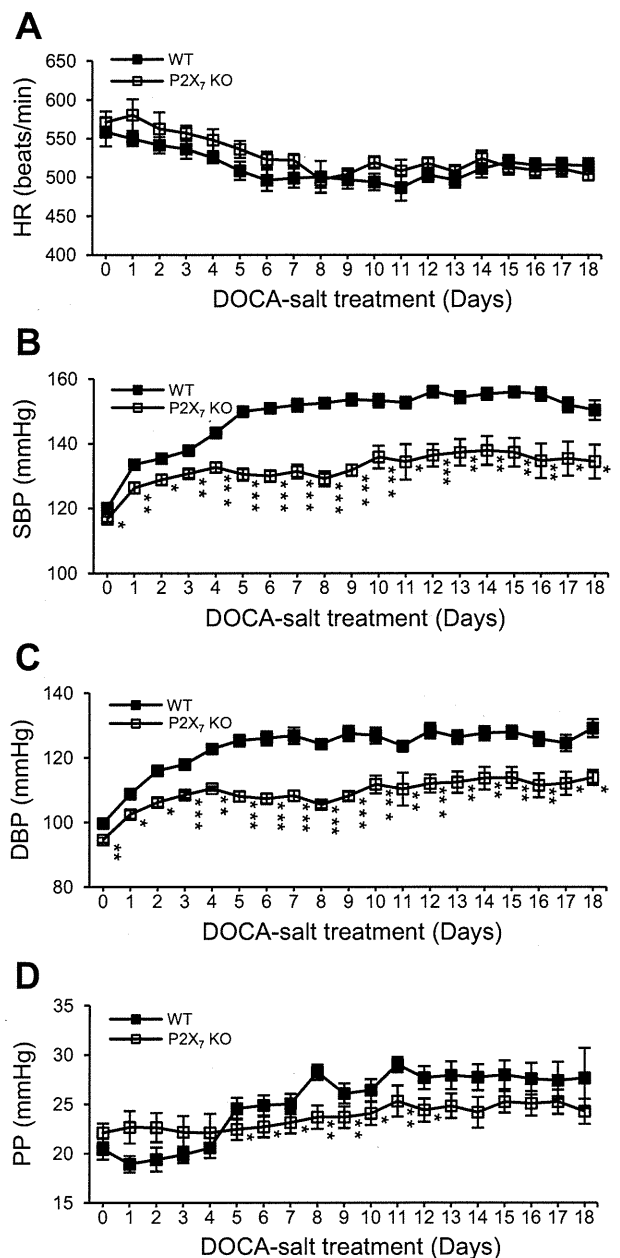


Fig. 1. Effects of P2X₇ deletion on heart rate (HR), systolic blood pressure (SBP), diastolic blood pressure (DBP), and pulse pressure (PP) in response to DOCA-salt treatment. HR (A), SBP (B), DBP (C), and PP (D) in WT ($n = 8$) and P2X₇-deficient (P2X₇ KO; $n = 8$) mice were measured for 24 h with a telemetry system. SBP, DBP, and PP were lower in P2X₇ KO mice than in WT mice; average SBP, DBP, and PP were also lower in P2X₇ KO mice than in WT mice. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, significantly different from WT mice (unpaired Student's t -test).

(WT and P2X₇ KO, $n = 10$, fed a normal diet) were injected intraperitoneally with 3% sterile thioglycollate medium (BD Diagnostic Systems, Sparks, MD), and 4 days later the peritoneal exudate corpuscles were collected by lavage with PBS. Cell suspensions were seeded in 12-well cell culture plates (2×10^6 cells/well) in RPMI 1640 medium (Nacalai Tesque, Kyoto, Japan) containing 10% fetal bovine serum (Thermo Scientific Hyclone, Logan, UT) and 2 mM L-glutamine (GIBCO-BRL, Grand Island, NY) (2 wells for each sample). After 4 h of incubation at 37°C in a 5% CO₂ atmosphere, nonadherent cells were removed with fresh RPMI 1640 medium. After incubation for 48 h at 37°C, to accumulate IL-1 β in macrophages, these cells were then primed with 10 ng/ml lipopolysaccharide (LPS, recombinant source from *Escherichia coli*, serotype O111: B4; Sigma-Aldrich, Poole, UK) for 24 h at 37°C. To assess IL-1 β release, the LPS-stimulated cells were stimulated for 6 h with 100 μ M BzATP, a P2X₇ agonist that is a mixture of 2'- and 3'-*O*-(4-benzoylbenzoyl)adenosine 5'-triphosphate (Sigma, St. Louis, MO). Cells treated with vehicle (PBS) served as negative controls. The media were then transferred to new tubes and centrifuged at 14,000 rpm for 10 min at 4°C to remove any nonadherent cells. The clear supernatants were stored at -80°C until use. Mouse IL-1 β was measured in duplicate with an enzyme-linked immunosorbent assay kit (Thermo Scientific, Rockford, IL).

Spectrophotometric assay for determination of total serum antioxidant activity. To evaluate the ability of reactive oxygen species, we measured the total serum antioxidant activity by using a total antioxidant power colorimetric microplate assay kit (Oxford Biomedical Research, Oxford, MI). This assay measures reductive capacity by detecting the reduction of Cu²⁺ to Cu⁺.

Statistical analysis. Values are expressed as means \pm SE. Statistical analyses were performed with JMP (SAS Institute, Cary, NC). Analysis of variance (ANOVA) was used to estimate differences

among groups, and the significance of differences was tested with Student's *t*-test.

RESULTS

HR and blood pressure. Baseline values of HR and PP (before DOCA-salt treatment) in the WT mice were not significantly different from those in the P2X₇ KO mice. However, the basal SBP and DBP values were significantly different between the two groups, although the difference was small [SBP: 120.1 ± 1.2 vs. 116.7 ± 0.8 mmHg ($P < 0.05$), DBP: 99.7 ± 0.1 vs. 94.6 ± 0.8 mmHg ($P < 0.01$), WT vs. P2X₇ KO, respectively] (Fig. 1).

There were no significant differences in HR between WT and P2X₇ KO mice during the DOCA-salt treatment period. Blood pressure of the WT mice increased progressively during the DOCA-salt treatment period, peaking on day 5 (mean SBP and DBP from days 5–18: 153.2 ± 1.9 and 126.4 ± 1.9 mmHg, respectively). DOCA-salt treatment resulted in steady markedly high levels of SBP, DBP, and PP from day 5 in the WT mice; however, these parameters only increased marginally in the P2X₇ KO mice ($P < 0.0001$ with ANOVA; Fig. 1).

Renal function. Glomerular barrier function was determined by assessing urinary albumin concentrations during the DOCA-salt treatment period (Fig. 2A), and renal filtration function was evaluated by measuring C_{Cr} on day 18 of the treatment (Fig. 2B).

Baseline urinary albumin levels were similar in WT and P2X₇ KO mice. Urinary albumin levels increased progressively during the DOCA-salt treatment period in the WT mice.

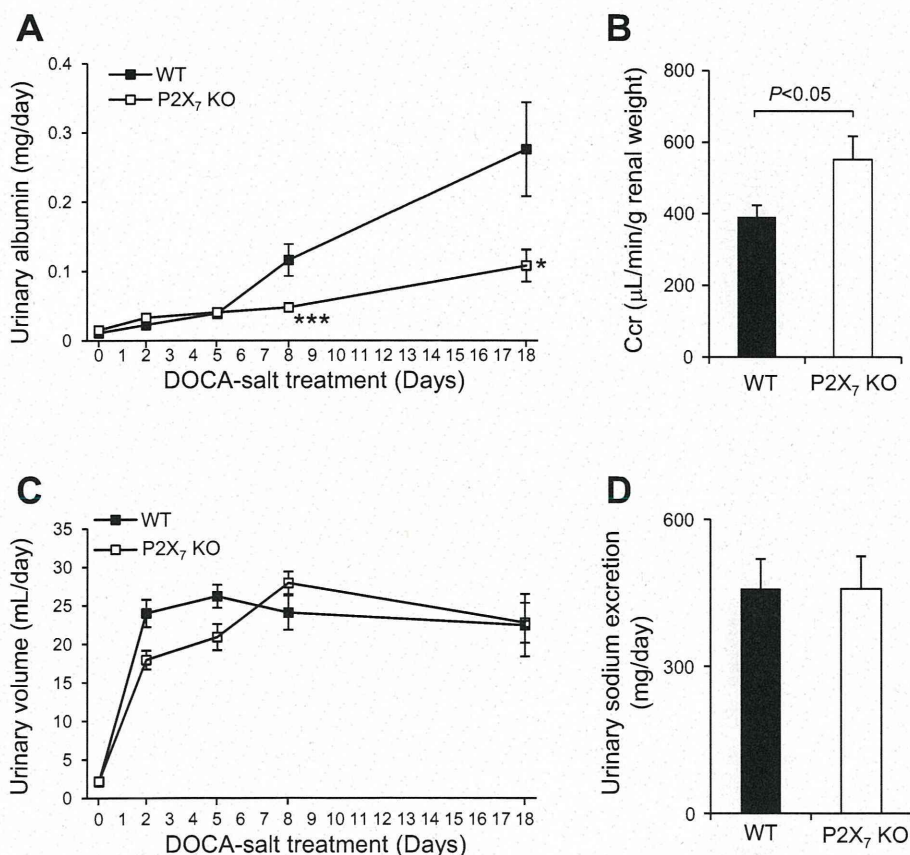


Fig. 2. Effects of P2X₇ deletion on renal function in response to DOCA-salt treatment: urinary albumin (A), creatinine clearance (C_{Cr}; B), urinary volume (C), and urinary sodium excretion (D) in WT and P2X₇ KO mice. $n = 10$ for WT and $n = 8$ for P2X₇ KO mice in A and C; $n = 18$ for WT and $n = 16$ for P2X₇ KO mice in B and D. * $P < 0.05$, *** $P < 0.001$, significantly different from WT mice (unpaired Student's *t*-test).

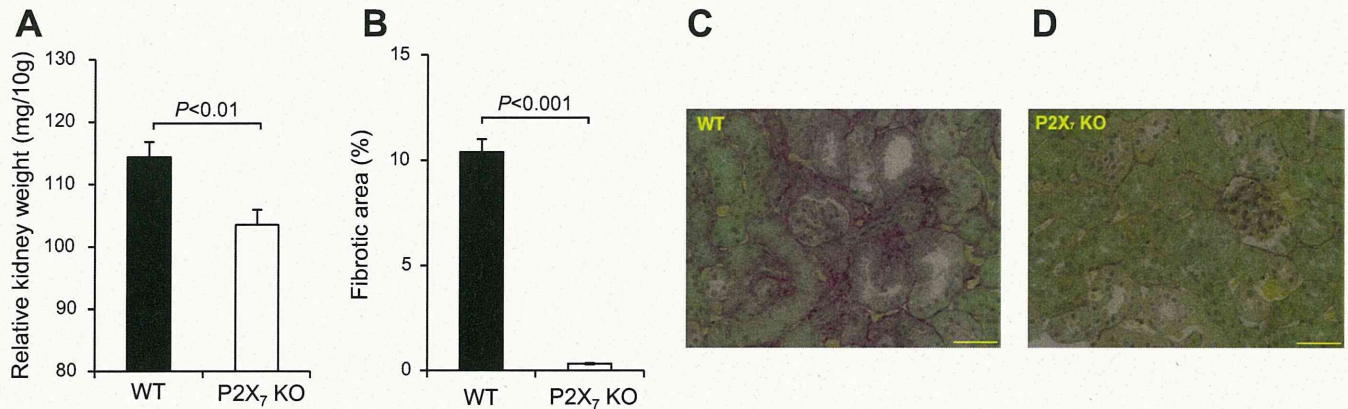


Fig. 3. Effects on interstitial fibrosis due to DOCA-salt hypertension in P2X₇ KO mice. **A**: relative renal weight in WT ($n = 18$) and P2X₇ KO ($n = 16$) mice; values represent renal organ weight per 10 g body weight. **B**: % of interstitial fibrosis area 18 days after DOCA-salt treatment in WT ($n = 8$) and P2X₇ KO ($n = 8$) mice. **C** and **D**: representative images of the kidneys of WT (**C**) and P2X₇ KO (**D**) mice stained with Sirius red show interstitial fibrosis (red-colored area). WT mice exhibited significantly larger areas of interstitial fibrosis than P2X₇ KO mice. Scale bars, 50 μ m.

However, on *days 8 and 18*, the urinary albumin levels in the P2X₇ KO mice were lower than those in the WT mice ($P < 0.001$ and $P < 0.05$ on *days 8 and 18*, respectively). On *day 18*, the urinary albumin measurements of the P2X₇ KO and WT mice were 0.11 ± 0.07 and 0.28 ± 0.07 mg/day, respectively. On *day 18*, C_{Cr} was significantly higher in the P2X₇ KO mice than in the WT mice ($P < 0.05$; Fig. 2B). However, there were no significant differences in urinary volume (Fig. 2C) or sodium excretion (Fig. 2D) between WT and P2X₇ KO mice.

On *day 18*, the renal weight of the P2X₇ KO mice was lower than that of the WT mice ($P < 0.01$; Fig. 3A). Histological examination detected renal interstitial fibrosis in both WT and

P2X₇ KO mice (Fig. 3, C and D); however, it was attenuated in P2X₇ KO mice compared with WT mice on *day 18* of DOCA-salt treatment ($P < 0.001$; Fig. 3B).

P2X₇ expression response to DOCA-salt treatment. Immunohistochemical analysis of renal P2X₇ revealed that the number of P2X₇-positive cells increased significantly in WT mice after DOCA-salt treatment (Fig. 4C) compared with that in untreated WT mice (Fig. 4A). However, P2X₇-positive cells were undetectable under all conditions in P2X₇ KO mice (Fig. 4, B and D). The numbers of P2X₇-positive cells are shown in Fig. 4E. Similar results were obtained for the expression of P2X₇ mRNA ($P < 0.05$; Fig. 4F).

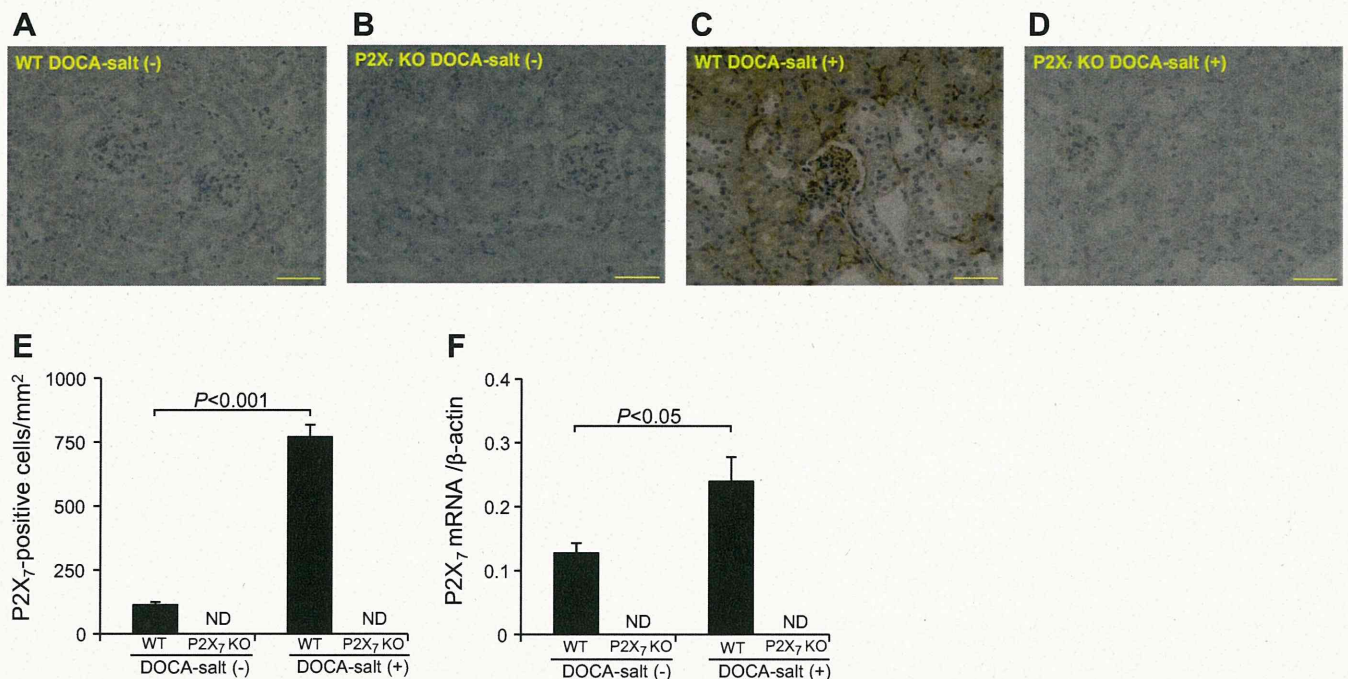


Fig. 4. Effects of DOCA-salt treatment on P2X₇ expression in the kidneys. The number of P2X₇-positive cells increased significantly in DOCA-salt treated WT mice (**C**) compared with untreated WT mice (**A**). No P2X₇-positive cells were detected in either untreated (**B**) or DOCA-salt treated (**D**) P2X₇ KO mice. Mice without DOCA-salt treatment are designated DOCA-salt(-); DOCA-salt treatment after 18 days is designated DOCA-salt(+). **E**: average number of P2X₇-positive cells per mm² in WT and P2X₇ KO mice with and without DOCA-salt treatment. **F**: expression levels of P2X₇ mRNA in WT and P2X₇ KO mice with and without DOCA-salt treatment. Values are ratios relative to those of β -actin. ND, not detected ($n = 8$ for WT and P2X₇ KO mice). Scale bars, 50 μ m.

Renal inflammatory response expression after DOCA-salt treatment. Immunohistochemical analysis of the kidneys revealed that infiltration of macrophages (F4/80-positive cells), T cells (CD3-positive cells), B cells (CD20-positive cells), and leukocytes (CD45-positive cells) was also markedly attenuated in P2X₇ KO mice compared with that in WT mice ($P < 0.001$; Fig. 5).

Expression site studies in consecutive sections were carried out to determine the relationship between P2X₇ and macrophages in the kidneys. P2X₇ receptor immunoreactivity was colocalized with positive macrophages (Fig. 6).

To determine the relationship between P2X₇ receptors and podocytes, colocalization studies of P2X₇ and podocytes in consecutive sections were performed. P2X₇ receptor immuno-

reactivity was colocalized with positive synaptopodin (a podocyte marker) in the glomeruli (Fig. 7).

IL-1 β release from WT and P2X₇ KO mouse macrophages. To confirm the capacity for IL-1 β release mediated by P2X₇ receptor stimulation, peritoneal macrophages from WT and P2X₇ KO mice were primed with LPS and then stimulated with the P2X₇ agonist BzATP. In the presence of LPS only, IL-1 β was released from neither WT nor P2X₇ KO mouse macrophages. However, in the presence of LPS and BzATP, IL-1 β was released from the WT mouse macrophages but not from the P2X₇ KO mouse macrophages ($P < 0.001$; Fig. 8).

Effects of DOCA-salt treatment on COX-2 expression in the kidneys. Immunohistochemical examination of the kidney tissues revealed that, on day 18 of DOCA-salt treatment, the

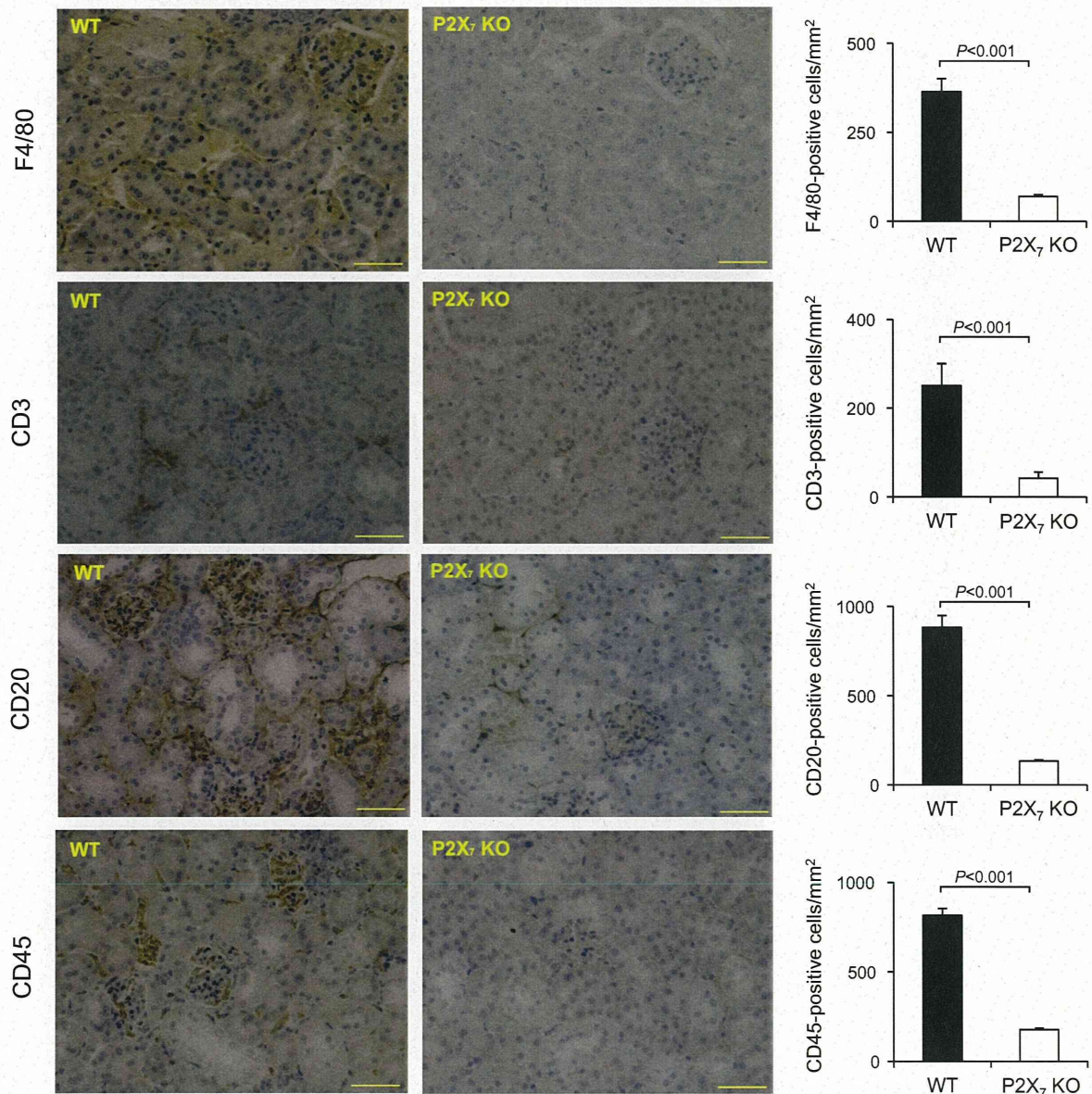


Fig. 5. Effects of DOCA-salt treatment on immune cell infiltration in the kidneys. The infiltration of macrophages (F4/80-positive cells), T cells (CD3-positive cells), B cells (CD20-positive cells), and leukocytes (CD45-positive cells) was measured by immunohistochemistry. There were significantly greater numbers of these immune cells in the kidneys of WT mice than those of P2X₇ KO mice. The average numbers of immune cells per mm² on day 18 after DOCA-salt treatment in WT and P2X₇ KO mice are also shown. However, immune cell infiltration was significantly attenuated in P2X₇ KO mice compared with WT mice ($n = 8$ for WT and P2X₇ KO mice). Scale bars, 50 μ m.

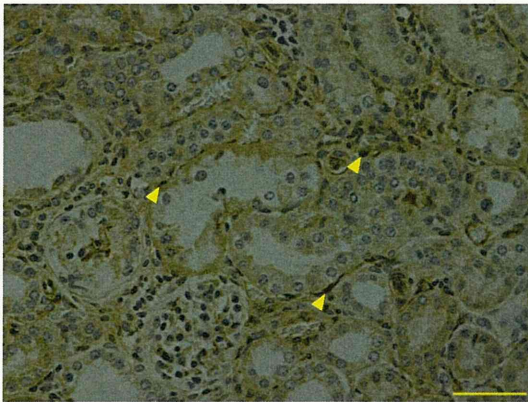
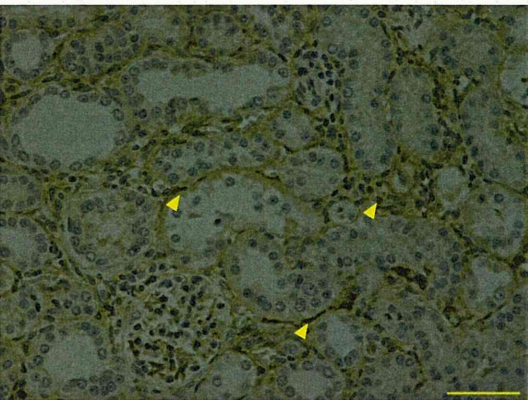
A P2X₇**B F4/80**

Fig. 6. Typical photographs of P2X₇ and F4/80 (a macrophage marker) colocalization in consecutive renal sections from WT mice. Immunohistochemical examination identified P2X₇ and macrophages by positive staining with anti-P2X₇ (A) and anti-F4/80 (B) antibodies, respectively. Macrophages coexpressed with P2X₇ are shown by arrowheads. Scale bars, 50 μ m.

expression levels of COX-2 protein in the kidneys of P2X₇ KO mice were markedly lower than the corresponding levels in WT mice (Fig. 9).

Effects of DOCA-salt treatment on total serum antioxidant activity. To evaluate the ability of reactive oxygen species, the total serum antioxidant activity (expressed as copper reducing equivalents) was measured. The level of activity was significantly higher in P2X₇ KO mice than in WT mice on *day 18* of DOCA-salt treatment (Fig. 10).

DISCUSSION

The P2X₇ receptor plays a key role in the modulation of inflammatory processes (1, 7, 15, 16). Indeed, the P2X₇ receptor is involved in various hypertension (12, 19) and renal (10,

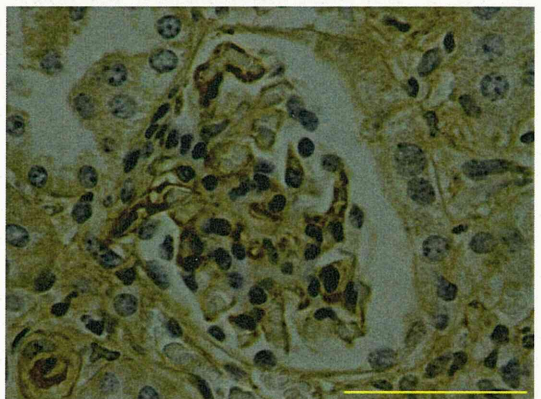
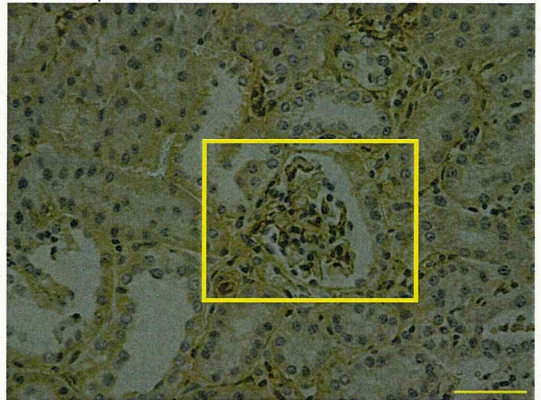
A P2X₇**B Synaptopodin**

Fig. 7. Typical photographs of P2X₇ and synaptopodin (a podocyte marker) colocalization in consecutive renal sections from WT mice. Immunohistochemical examination identified P2X₇ and macrophages by positive staining with anti-P2X₇ (A) and anti-synaptopodin (B) antibodies, respectively. Podocytes coexpressed with P2X₇ are shown. *Bottom*: magnified photographs of rectangular areas shown at *top*. Scale bars, 50 μ m.

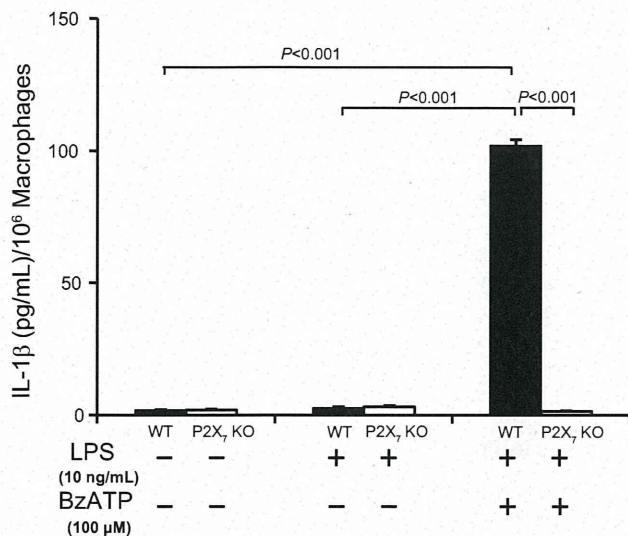


Fig. 8. IL-1 β release from peritoneal macrophages of WT and P2X₇ KO mice, mediated by P2X₇ receptor stimulation. Peritoneal macrophages from WT and P2X₇ KO mice were primed with LPS and then stimulated with the P2X₇ agonist BzATP. In the presence of LPS only, IL-1 β was released from neither WT nor P2X₇ KO mouse macrophages. However, IL-1 β was released from the WT mouse macrophages but not from the P2X₇ KO mouse macrophages in response to LPS + BzATP treatment ($n = 10$ for WT and P2X₇ KO mice).

13, 29, 30) diseases. However, its functional role in hypertension and renal injury remains unclear.

In the present study, deletion of the P2X₇ gene ameliorated hypertension and renal function by reducing renal injury and inflammation. Specifically, renal injury was attenuated as reflected by reduced urinary albumin levels, renal interstitial fibrosis, and increased C_{Cr} levels. In addition, renal inflammation was also reduced, as determined by decreased inflammatory cell infiltration (i.e., macrophages, T cells, B cells, and leukocytes). Moreover, IL-1 β was released by the WT mouse macrophages but not by the P2X₇ KO mouse macrophages. These results strongly suggest that P2X₇ is involved in hypertension and renal injury via renal inflammatory responses.

Although only minimally, the lower baseline blood pressure of the P2X₇ KO mice was significantly different from that of the WT mice. This suggests that the P2X₇ receptor contributes minimally to baseline blood pressure. Renal P2X₇ mRNA and protein expression were significantly upregulated in WT mice after DOCA-salt treatment; this upregulation may be related to

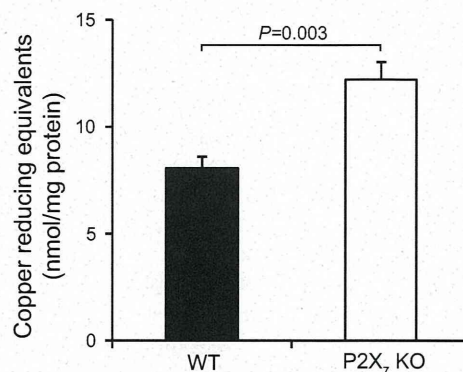


Fig. 10. Effects of DOCA-salt treatment on total serum antioxidant activity. Total serum antioxidant activity was measured with a total antioxidant power kit. The level of activity (expressed as copper reducing equivalents) was significantly higher in P2X₇ KO mice than that in WT mice on day 18 of DOCA-salt treatment ($n = 10$ for WT and P2X₇ KO mice).

elevated blood pressure in WT mice. Furthermore, DOCA-salt treatment elevated the blood pressure of WT mice, but this elevation was significantly attenuated in P2X₇ KO mice.

Chronic hypertension is a major risk factor for end-stage renal disease (2, 4). Furthermore, there is a greater incidence of end-stage renal disease in patients with salt-sensitive hypertension (22). In the present study, urinary sodium excretion was not significantly different between WT and P2X₇ KO mice, suggesting that the same amount of sodium is excreted in both types of mice after high salt intake. Urinary albumin excretion increased significantly in the WT mice after DOCA-salt treatment, but this increase was strongly attenuated in the P2X₇ KO mice. C_{Cr} levels were much higher in the P2X₇ KO mice than in the WT mice, and the renal weight of the P2X₇ KO mice was lower than that of the WT mice. In P2X₇ KO mice, renal injury was alleviated, a further increase in renal weight was inhibited, and C_{Cr} levels also increased because of improved renal function. Therefore, C_{Cr} levels are inversely related to renal weight. These results suggest that P2X₇ receptor deficiency protects renal function during DOCA-salt hypertension.

We aimed to determine how P2X₇ affects DOCA-salt hypertension and renal injury. Stimulation of the P2X₇ receptor is reported to cause the release of inflammatory cytokines from macrophages and lymphocytes (1, 17, 20, 32, 33). In addition, macrophage and T-cell infiltration in the kidneys is regarded as a key event in renal injury, leading to renal fibrosis and

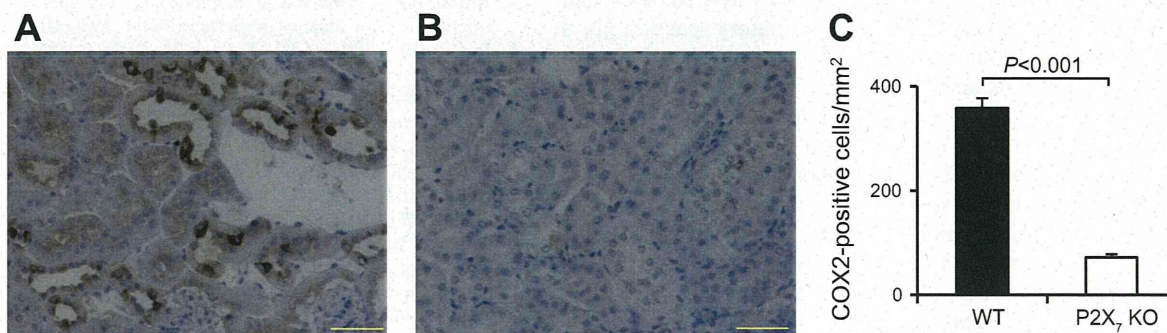


Fig. 9. Effects of DOCA-salt treatment on COX-2 expression in the kidneys. A and B: positive cell numbers of COX-2 were significantly higher in WT mice (A) than in P2X₇ KO mice (B) on day 18 of DOCA-salt treatment. C: average positive cell number of COX-2 per mm² in WT and P2X₇ KO mice on day 18 of DOCA-salt treatment. ($n = 8$ for WT and P2X₇ KO mice). Scale bars, 50 μ m.

proteinuria (7, 15, 16). Recent reports also suggest that renal infiltration involving immune cells plays a role in the pathogenesis of hypertension (5, 18, 21). Therefore, in the present study, to address the aforementioned issue, the infiltration of immune cells (i.e., macrophages, T cells, B cells, and leukocytes) was measured by immunohistochemistry. Immune cell infiltration increased significantly in the WT mice after DOCA-salt treatment, but this increase was significantly smaller in the P2X₇ KO mice. Furthermore, the colocalization of P2X₇ and F4/80 (a macrophage marker) was observed in consecutive renal sections. These results suggest that renal P2X₇ expression may be correlated with the inflammatory pathway via the stimulation of immune cells.

Although immune cells are reported to play a deleterious role in both the hypertension and renal injury associated with DOCA-salt hypertension (5), the mechanisms by which immune cells are involved in hypertension and renal injury are unclear. We confirmed the capacity of the peritoneal macrophages of WT and P2X₇ KO mice to release IL-1 β mediated by P2X₇ receptor stimulation. After LPS and BzATP stimulation, IL-1 β was released from WT mouse macrophages but not from P2X₇ KO mouse macrophages. These results suggest that the release of the inflammatory cytokine IL-1 β by macrophages is related to the P2X₇ receptor. Inflammatory responses were attenuated in immune cells deficient in P2X₇, which subsequently attenuated inflammatory responses preventing renal injury, which in turn led to lower blood pressure. Therefore, this study revealed that the P2X₇ receptors of immune cells play important roles in renal injury and hypertension. The activation of macrophages and T cells in P2X₇ KO mice is reported to be lower than that in WT mice (3, 28). The results of these reports also support our hypothesis. In addition, P2X₇ deficiency also affected downstream effectors of IL-1 β signaling, such as COX-2 and reactive oxygen species.

Initially, DOCA-salt treatment increased blood pressure slightly; this initial slight increase in blood pressure could induce minimal renal injury, which may have increased the inflammatory reaction. In turn, the inflammatory reaction may be related to the P2X₇ pathway of immune cells, which could then lead to progressively worsening renal function, resulting in moderate hypertension. Moreover, such renal injury is likely to initiate a vicious cycle of hypertension, resulting in greater nephron loss and blood pressure. The P2X₇ receptor did not affect the initial slight increase in blood pressure because blood pressure until *day 4* was slightly elevated in both P2X₇ KO and WT mice after DOCA-salt treatment. Therefore, the P2X₇ receptor attenuates only the vicious cycle of hypertension and renal injury via immune cells. The inflammatory reaction plays an important role via the P2X₇ pathway of immune cells in this vicious cycle between renal injury and hypertension.

The causes of hypertension and renal injury are very complicated. A recent report suggests that the P2X₇ receptor is involved in thrombosis (9). As thrombosis of the kidneys may beget renal injury, it is also a possible cause of hypertension and renal injury. Furthermore, several studies report that the P2X₇ receptor is expressed in glomerular mesangial cells (11, 24, 30), podocytes (8, 30), and medullary collecting duct cells (25). We also found that the P2X₇ receptor is expressed in podocytes by examining the immunohistochemistry of P2X₇-synaptopodin (a podocyte marker) colocalization. The P2X₇ receptor of these cells might also be involved in the regulation

of blood pressure and renal function. However, further studies are needed to investigate these speculations.

In conclusion, our results demonstrate the following: elevated P2X₇ levels evoke sustained renal inflammation, which could lead to the development of hypertension and renal injury; P2X₇ deficiency prevents the development of hypertension and renal injury; and there is a positive relationship between the P2X₇ gene and hypertension. Our results suggest that P2X₇ plays important roles in the development and progression of hypertension and renal injury. Moreover, the P2X₇ receptor may be an important novel therapeutic target in hypertension-induced renal injury. This study contributes to a better understanding of mechanisms, prevention, and therapies for hypertension and renal injury.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: X.J., Y.N., and N.I. conception and design of research; X.J., Y.N., H.W., K.E., and X.M. performed experiments; X.J., Y.N., H.W., K.E., and X.M. analyzed data; X.J., Y.N., and N.I. interpreted results of experiments; X.J., Y.N., and N.I. prepared figures; X.J., Y.N., and N.I. drafted manuscript; X.J., Y.N., and N.I. edited and revised manuscript; X.J., Y.N., and N.I. approved final version of manuscript.

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