

β -estradiol alleviates hypertension- and concanavalin A-mediated inflammatory responses via modulation of connexins in peripheral blood lymphocytes

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Abstract. Gap junctions (GJs) formed by connexins (Cxs) in T lymphocytes have been reported to have important roles in the T lymphocyte-driven inflammatory response and hypertension-mediated inflammation. Estrogen has a protective effect on cardiovascular diseases, including hypertension and it attenuates excessive inflammatory responses in certain autoimmune diseases. However, the mechanisms involved in regulating the pro-inflammatory response are complex and poorly understood. The current study investigated whether β -estradiol suppresses hypertension and pro-inflammatory stimuli-mediated inflammatory responses by regulating Cxs and Cx-mediated GJs in peripheral blood lymphocytes. Male, 16-week-old spontaneously hypertensive rats (SHR) and Wistar-Kyoto rats (WKY) rats were randomly divided into the following three groups: WKY rats, vehicle (saline)-treated SHRs, and β -estradiol (20 μ g/kg/day)-treated SHRs. β -estradiol was administered subcutaneously for 5 weeks. Hematoxylin and eosin staining was performed to evaluate target organ injury. Flow cytometry and ELISA were used to measure

the populations of T lymphocyte subtypes in the peripheral blood, and expression of Cx40/Cx43 in T cell subtypes, and pro-inflammation cytokines levels, respectively. ELISA, a dye transfer technique, immunofluorescence and immunoblotting were used to analyze the effect of β -estradiol on pro-inflammatory cytokine secretion, Cx-mediated GJs and the expression of Cxs in concanavalin A (Con A)-stimulated peripheral blood lymphocytes isolated from WKY rat. β -estradiol significantly decreased blood pressure and inhibited hypertension-induced target organ injury in SHRs. Additionally, β -estradiol treatment significantly improved the immune homeostasis of SHRs, as demonstrated by the decreased percentage of cluster of differentiation (CD)4⁺/CD8⁺ T-cell subset ratio, reduced serum levels of pro-inflammatory cytokines and increased the percentage of CD4⁺CD25⁺ T cells. β -estradiol also markedly reduced the expression of Cx40/Cx43 in T lymphocytes from SHRs. *In vitro*, β -estradiol significantly suppressed the production of pro-inflammatory cytokines, reduced communication via Cx-mediated gap junctions and decreased the expression of Cx40/Cx43 in Con A-stimulated lymphocytes. These results indicate that β -estradiol attenuates inflammation and end organ damage in hypertension, which may be partially mediated via downregulated expression of Cxs and reduced function of Cx-mediated GJ.

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Introduction

Hypertension is a worldwide epidemic and global health problem, and is one of the most important risk factors for cardiovascular disease events, which are a major cause of morbidity and mortality (1-3). In recent years, the frequency of hypertension has been gradually increasing in China (4). It is reported that ~300 million people in China will be living with hypertension by 2017 and the morbidity rate increases gradually with age (4). A large number of clinical and experimental investigations from numerous laboratories around the world have demonstrated that chronic low-grade inflammation

and the adaptive immune response make important contributions to the pathogenesis of various forms of systemic hypertension (5), other cardiovascular diseases (6), and renal disease (1). However, there is mounting evidence, supporting the concept that hypertensive stimuli induce activation of T lymphocytes and infiltration of activated T lymphocytes into target organs, including the peripheral blood vessels and kidney (7); with Dahl-salt sensitive rats and spontaneously hypertensive rats (SHRs) as example models (8). When activated, effector T lymphocytes contribute to blood pressure elevation by exacerbating vascular remodeling and chronic kidney injury directly via the release of pro-inflammatory cytokines, including tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6) (8). Furthermore, to a large extent, the severity of inflammation is determined by imbalances between pro-inflammatory responses of effector T cells and anti-inflammatory responses of regulatory T lymphocytes (Tregs) (9). Although the role of T lymphocytes in hypertension-mediated inflammation is clearly defined by a large body of experimental data, the evidence that inflammation and an adaptive immune response are induced by hypertension is rather limited. The authors' laboratory has demonstrated that gap junctional communication via connexins (Cx) in peripheral blood lymphocytes of SHRs (10) and hypertensive patients (11) is involved in the hypertension-mediated inflammatory response, and that Cx expression is positively correlated with the proliferation of T lymphocyte and production of pro-inflammatory cytokines in the peripheral blood of patients with hypertension and SHRs (10-13). Data from the authors' laboratory and other groups have also revealed that inhibition of Cx43-mediated gap junctional communication can reduce the activation and proliferation of T lymphocytes and production of pro-inflammatory cytokines under various pro-inflammatory stimuli (10,11,14-16). Therefore, Cx-based channels may be novel potential targets for the treatment of hypertension-mediated inflammation.

Experimental and clinical studies have demonstrated that T-cell-targeted immunosuppressive drugs (including mycophenolate mofetil) and cytokine inhibitors reduce arterial pressure or/and ameliorate renal inflammation in hypertensive patients with rheumatoid arthritis and psoriasis, or in pharmacologically-induced (angiotensin II infusion and deoxycorticosterone acetate salt) or genetic rodent models of hypertension (Dahl salt-sensitive rats and SHRs) (1,3,9,17,18); however, these immunosuppressive therapies produce non-specific inhibition of the immune system, and therefore, may interfere with the normal generation of T lymphocytes, or result in unwanted and unsafe side-effects in hypertensive patients and animals (1,19). By contrast, estrogen replacement therapy using 17 β -estradiol reduces blood pressure (BP) via vasoprotective effects (20) and prevents a hypertension-mediated pro-inflammatory response by inhibiting the production of pro-inflammatory cytokines and stimulating the production of Tregs and IL-10 (21,22). Studies have also provided further evidence that increased pro-inflammatory and decreased anti-inflammatory status caused by ovarian hormone deficiency is associated with an increased frequency of hypertension in women and numerous animal models of hypertension (23); therefore, estrogen can keep women 'cardiovascularly younger' than men of the same age (24). However, there are clear gaps in the understanding of

the precise mechanisms underlying how estrogen regulates the adaptive immune response, BP and T lymphocyte profiles (23). In order to further expand the current knowledge of the cellular mechanisms of estrogen in preventing T lymphocyte-associated hypertension, the aim of the present study was to determine whether exogenous estrogen (β -estradiol) treatment can prevent hypertension-mediated inflammation and target organ damage by modulating Cx-mediated gap junctional intracellular communication (GJIC) in peripheral blood T lymphocytes. Identification of the cellular mechanisms underlying the regulatory role of estrogen in BP and hypertension-mediated inflammation may lead to specific treatment strategies that ultimately reduces the incidence of hypertension.

Materials and methods

Experimental animals and drug treatment. The 15-week-old male SHR and age-matched Wistar-Kyoto rats (WKY) rats from Vital Beijing River Laboratory Animal Technology Co., Ltd., (Beijing, China) were housed in plastic cages in a room with a relative humidity of 45-55%, temperature of 23 \pm 2°C and a 12-h light-dark cycle. Rats had free access to drinking water and food throughout the experiment. Prior to the onset of the experiment, all rats were allowed to train with and acclimate to the tail-cuff plethysmography procedure for 1 week. After 1 week, the systolic blood pressure was measured for 3 consecutive days to record the hypertension prior to starting the experiments. SHRs with a BP \geq 150 mmHg were used in subsequent experiments. The animal experimental procedures performed in the present study were approved by the Institutional Animal Care and Use Committees (permit number: A2016-047-03) of the Medical College of Shihezi University (Shihezi, China) and conducted in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health (25).

Male SHRs (16-week-old; n=12) with an initial body weight of 130-200 g were randomly divided into two groups: SHR group (n=6) and SHR + β -estradiol (E) group (n=6). Male WKY rats of the same age and body weight served as a control group (WKY group; n=6). SHR in the β -estradiol treatment group received a single subcutaneous injection of 20 μ g/kg/day β -estradiol (cat. no. E8875; \geq 98% pure; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) once daily at the same time each day from 16-21 weeks of age and rats in the other groups (WKY control group and the SHRs control group) were injected subcutaneously with the same volume of normal saline. The concentration of β -estradiol was selected based on the effective dosage in a previous study (26). Following treatment with drug for 5 weeks, the tail arterial BP of all rats was measured.

Measurement of tail arterial BP. Tail systolic arterial pressure was recorded in conscious and calm rats at 21 weeks old using a non-invasive tail-cuff apparatus (Chengdu Taimeng Software Co. Ltd., Chengdu, China) without heating, as described in the authors' previous study (13,27). The BP of each rat was taken as the average of at least three stable consecutive measurements following removal of outliers and any readings associated with

excess noise or animal movement on each occasion. Data are expressed as mmHg and calculated as the mean \pm standard error of the mean (SEM).

Morphology and histological analyses. At the end of the experiment, all rats were euthanized by an intraperitoneal injection of 50 mg/kg pentobarbital sodium (30 mg/l) at 21 weeks old. Kidneys and basilar arteries (BA) were harvested and fixed in 4% paraformaldehyde at 4°C for 48 h. The animals were sacrificed by decapitation under an overdose of pentobarbital (100 mg/kg) anesthesia at the end of the experiments. Fixed renal and vascular tissues from the different groups were dehydrated in graded concentrations of ethanol and imbedded in paraffin, then 5 μ m-thick sections were cut with a microtome. Kidney and BA sections were stained with hematoxylin and eosin as described in the authors' previous study (13). Histological observation of vascular remodeling and renal injury was performed using light microscopy (Olympus BX50 microscope; Olympus Corporation, Tokyo, Japan). A total of 1 tissue section was selected from each rat and at least 10 random non-overlapping fields (at x100 or 200 magnification) were imaged to observe the presence of inflammatory cell infiltration, thickness of the medial wall and endothelium injury in BA, and tubular dilation, glomerulus deformation and fibrosis in the kidneys.

Flow cytometric analysis of peripheral blood mononuclear cells (PBMCs). Following administration of β -estradiol or normal saline for 5 weeks, rats from each group were euthanized by an intraperitoneal injection of 50 mg/kg pentobarbital sodium and peripheral blood (5 ml) was collected from the abdominal aorta into a glass tube with EDTA, and PBMCs were separated and purified using a rat mononuclear cell isolation kit (cat. no. P8630; Beijing Solarbio Science & Technology, Beijing, China) and FACS™ Lysing solution (cat. no. 349202; BD Biosciences; Becton, Dickinson and Company, Franklin, Lakes, NJ, USA), according to the manufacturer's protocol. All overdose euthanized rats with pentobarbital sodium (100 mg/kg) were sacrificed by decapitation at the end of the experiments. The cell survival rate of isolated PBMCs was assessed by 0.4% Trypan blue staining for 10 min at room temperature. Subsequently, PBMCs ($>1 \times 10^6$ cells/ml) from the different groups were transferred to a tube and stained in PBS containing fluorescein isothiocyanate (FITC)-conjugated anti-rat CD3 (dilution 1:1,000; cat. no. 201403), allophycocyanin (APC)-conjugated anti-rat CD4 (dilution 1:400; cat. no. 201509), phycoerythrin (PE)-conjugated anti-rat CD8 (dilution 1:400; cat. no. 201705) and PE-conjugated anti-rat CD25 monoclonal antibodies (dilution 1:400; cat. no. 202105) (all antibodies from Biolegend, Inc., San Diego, CA, USA) for 30 min at 4°C in the dark. Isotype-matched, FITC-, APC-, and PE-conjugated monoclonal antibodies were used as negative controls. Following staining, cells were analyzed within 24 h using a flow cytometer (FACSsort; BD Pharmingen; BD Biosciences; Becton, Dickinson and Company) and data analysis was performed using BD CellQuest Pro software (version 2.0, system OS2; Becton, Dickinson and Company). Cluster of differentiation CD4⁺ T cells, CD8⁺ T cells and Treg cells were identified as double-positive stained cells (CD3⁺ and CD4⁺ or CD8⁺, and CD4⁺ and CD25⁺, respectively) and were

expressed as percentages of different T lymphocyte subpopulation.

Expression of Cx40 and Cx43 on CD4⁺ or CD8⁺ T cells was determined by flow cytometry as previously described, with minor modifications (11,13). Briefly, permeabilized PBMCs were incubated with anti-Cx40 monoclonal antibody (dilution 1:500; cat. no. sc-365107; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) or anti-Cx43 antibody (dilution 1:500; cat. no. sc-13558; Santa Cruz Biotechnology, Inc.) overnight at 4°C. Following washing, the PBMCs were incubated in FITC-labeled secondary antibody (dilution 1:500; cat. no. 405305; Biolegend, Inc.) and/or anti-CD4 and anti-CD8 antibodies. The expression of Cx40/Cx43 in different T lymphocyte subpopulations was analyzed using the two-color immunofluorescence flow cytometry method as described previously (11,13).

Measurements of cytokines in the serum. Cytokine levels in serum from the different groups were measured in duplicate using commercially available ELISA assay kits for TNF- α , IL-6 and IL-10 [TNF- α , cat. no. 70-EK382HS-96; IL-6, cat. no. 70-EK3062/2; and IL-10, cat. no. 70-EK3102/2; Hangzhou Multi Sciences (Lianke) Biotech Co., Ltd., Hangzhou, China] following the manufacturer's protocol. Results are expressed as pg/ml in each sample.

GJIC assay. The effect of β -estradiol on the GJIC between peripheral blood lymphocytes was measured using a calcein acetoxymethyl ester (calcein AM) transfer assay as described previously (10,11). Briefly, PBMCs (1×10^6 cells/ml) isolated from WKY rats were incubated in a 6-well plate with a 2.5 μ M solution of calcein AM (cat. no. 3099; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) or the lipophilic dye DiIC₁₈ (10 μ M; cat. no. D282; Invitrogen; Thermo Fisher Scientific, Inc.) for 30 min at 37°C in RPMI-1640 (cat. no. 11875085; Gibco; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (FBS; cat. no. SH30084; HyClone; GE Healthcare Life Sciences, Logan, UT, USA). Following incubation, these cells were washed five times with PBS containing 1% bovine serum albumin (BSA; cat. no. A8010; Beijing Solarbio Science & Technology Co., Ltd.). The donor cells (calcein⁺DiIC₁₈⁻) and the recipient cells (calcein⁻DiIC₁₈⁺) were cocultured at 1:10 ratio. Following seeding for 30 min, cocultured cells were incubated in RPMI-1640 medium supplemented with concanavalin A (Con A; 5 μ g/ml) and/or β -estradiol (10 nM) for 3 h (28). Following the indicated time of incubation, each group of cocultures were collected and suspended in PBS containing 1% BSA, and the transfer of calcein from donor cells to the recipient cells was analyzed by flow cytometry as described previously (11).

Cell culture and drug treatment of peripheral blood T lymphocytes. PBMCs isolated from WKY rats were cultured in RPMI-1640 growth medium (cat. no. 11875085; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% (v/v) heat-inactivated FBS (cat. no. SH30084; HyClone; GE Healthcare Life Sciences), 100 IU/ml penicillin and 100 IU/ml streptomycin (cat. no. P0781; Sigma-Aldrich; Merck KGaA) in an atmosphere of 5% CO₂ and 95% air at 37°C for

3 h. Following 3-h incubation, the cell viability of peripheral blood T lymphocytes was determined using trypan blue and cells were then adjusted to a concentration of 1×10^6 cells/ml in medium. The cultured cells were transferred into 6-well plates and pretreated with or without β -estradiol (10 nM) for 24 h, and then stimulated with $5 \mu\text{g/ml}$ Con A (cat. no. C5275; Sigma-Aldrich; Merck KGaA) for another 48 h at 37°C (humidified atmosphere, 5% CO_2). Untreated controls were cultured under the same conditions without Con A and β -estradiol. Following drug treatment for the indicated times, all cells and culture supernatants collected were used to analyze the expression of Cxs (Cx40 and Cx43) and the concentration of cytokines (TNF- α and IL-6) by immunofluorescence/immunoblotting and ELISA, respectively.

Immunofluorescence staining. Peripheral blood lymphocytes (1×10^5 cells/ml) isolated from WKY rats were incubated with or without Con A and/or β -estradiol in a 5% CO_2 and 95% air atmosphere for the indicated duration at 37°C . Subsequently, cells were washed with PBS then fixed in PBS containing 4% paraformaldehyde for 30 min at room temperature. Lymphocytes were washed with PBS three times and permeabilized with 0.5% Triton X-100/0.5% FBS for 10 min. Following washing with PBS, each well was blocked with 1% BSA/PBS for 1 h at room temperature. Following blocking, peripheral blood lymphocytes were incubated overnight at 4°C with anti-Cx40 monoclonal antibody (dilution 1:500; cat. no. sc-365107; Santa Cruz Biotechnology, Inc.) or anti-Cx43 antibody (dilution 1:500; cat. no. sc-13558; Santa Cruz Biotechnology, Inc.) in 1% BSA/PBS. The cells were washed thoroughly, and the bound primary antibody was detected by incubating the cells with secondary FITC-conjugated goat anti-mouse IgG (dilution 1:100; cat. no. ZF0312; OriGene Technologies, Inc., Rockville, MD, USA) for 2 h at room temperature in the dark. Subsequently, cells were counterstained with $10 \mu\text{g/ml}$ DAPI (cat. no. C0065; Beijing Solarbio Science & Technology Co., Ltd.) at 37°C for 2 min to visualize the nucleus. Fluorescence images were captured using a laser scanning confocal microscope (Zeiss LSM510; Carl Zeiss AG, Oberkochen, Germany) with a 63x oil immersion objective (numerical aperture, 1.40). Adobe Photoshop software (version 4.0; Adobe Systems, Inc., San Jose, CA, USA) was used to adjust the contrast of the images and to compose and overlay the images. Semiquantitative analysis of the mean fluorescence intensities of Cx40 and Cx43 was performed using ImageJ software (version 1.52a; National Institutes of Health, Bethesda, MD, USA). A total of 50 peripheral blood lymphocytes from each group in ~ 25 fields were evaluated in at least three experimental repeats.

Immunoblotting. Peripheral blood lymphocytes (1×10^6 cells/well) from WKY rats were transferred to 6-well plates. Following Con A ($5 \mu\text{g/ml}$) or/and β -estradiol (10 nM) treatment for the indicated time, the expression levels of Cx40 and Cx43 in PBMCs were determined by immunoblot analysis as previously described (11,13). PBMCs were lysed with ice-cold lysis buffer (25 mM bicine, 150 mM sodium chloride, pH 7.6; cat. no. 78510; Pierce; Thermo Fisher Scientific, Inc.) containing 1 mM phenylmethylsulfonyl fluoride for 30 min at 4°C . Lysed lymphocytes were sonicated and centrifuged at $10,000 \times g$ for 20 min at 4°C . The supernatant was collected

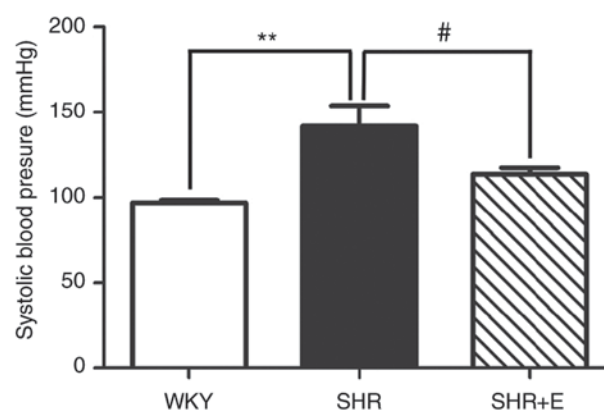


Figure 1. Effects of β -estradiol supplement on tail systolic arterial pressure in SHR. Tail systolic arterial pressure of the SHR group significantly increased in comparison with the WKY group. Compared with SHR without β -estradiol treatment, chronic subcutaneous injection of $20 \mu\text{g/kg/day}$ β -estradiol for 5 weeks significantly attenuated hypertension in the SHR + E group. Values are presented as the mean \pm standard error, $n=6$. ** $P<0.01$ ($P=0.0061$), WKY vs. SHR and # $P<0.05$ ($P=0.0447$), SHR vs. SHR + E. SHR, spontaneously hypertensive rat; WKY, Wistar-Kyoto rats; SHR + E, SHR + β -estradiol.

and the protein concentration was determined with a Bradford protein assay kit (cat. no. GK5021; Genaray Biotech Co., Ltd., Shanghai, China). Each lane was loaded with an equal amount of protein ($25 \mu\text{g/lane}$) and separated by SDS-PAGE on 10% gels. Separated proteins were transferred to a polyvinylidene fluoride membrane as described previously (11,13). The following antibodies were used for western blot analysis: Mouse monoclonal anti-Cx40 antibody (1:1,000; overnight incubation at 4°C ; cat. no. sc-365107; Santa Cruz Biotechnology, Inc.), mouse monoclonal anti-Cx43 antibody (1:1,000; overnight incubation at 4°C ; cat. no. sc-13558; Santa Cruz Biotechnology, Inc.), mouse monoclonal anti- β -actin antibody (1:1,000; overnight incubation at 4°C ; cat. no. TA-09; OriGene Technologies, Inc.) and horseradish peroxidase-conjugated goat anti-mouse secondary antibody (1:10,000; incubation for 1.5 h at room temperature; cat. no. ZB-5305; OriGene Technologies, Inc.). Cx signals were visualized using an enhanced chemiluminescence reagent (cat. no. RPN2109; GE Healthcare Life Sciences) and quantified using Quantity One analysis software (version 4.6.8, Bio-Rad Laboratories, Inc., Hercules, CA, USA). β -actin was used as an internal standard to normalize the protein levels of Cxs in each sample.

Statistical analysis. All experimental data are presented as the mean \pm SEM. Statistical analysis was performed using GraphPad Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA). Data with more than two groups were compared using one-way analysis of variance, followed by a post-hoc test (Tukey's multiple comparison test). All of the experiments were performed at least three times independently. $P<0.05$ was considered to indicate a statistically significant difference.

Results

β -estradiol reduces systolic arterial pressure in SHRs. Tail systolic arterial pressure was determined using tail-cuff plethysmography in all rats at 21 weeks. Compared with the

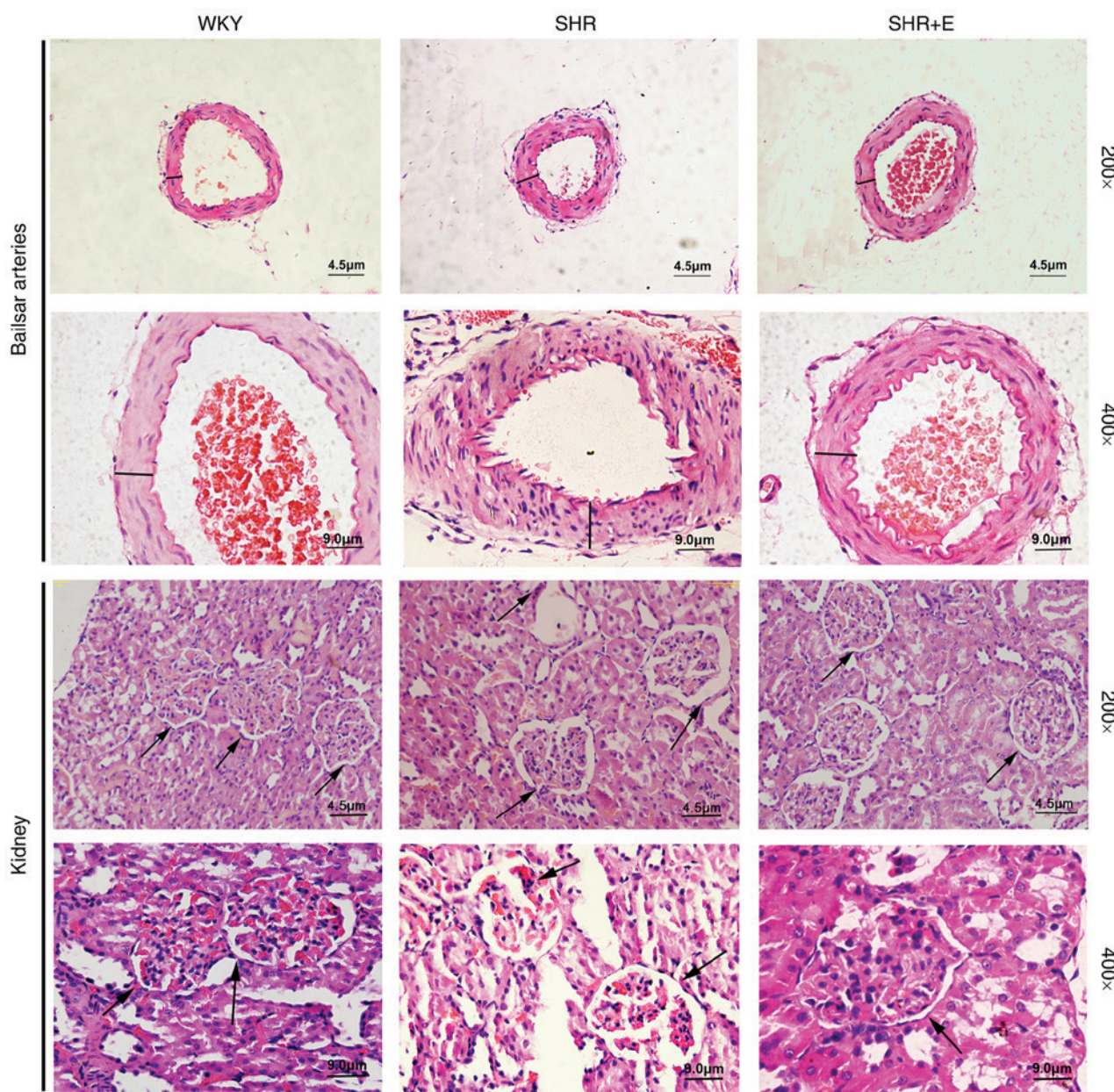


Figure 2. β -estradiol supplement attenuates vascular remodeling of BA, target organ damage and inflammatory cell infiltration in BA and kidney tissues of SHR. Representative pictures of hematoxylin and eosin staining of renal and arterial sections indicated the protective role of β -estradiol on target organ damage. The two highlighted black lines represent the label of basilar arteries and kidney. The arrows indicate atrophy of the glomerulus, infiltration of inflammatory cell into part of renal interstitium. Magnification, x200 (scale bar, 4.5 μ m) or x400 (scale bar, 9.0 μ m). n=6. BA, basilar arteries; SHR, spontaneously hypertensive rat; WKY, Wistar-Kyoto rats; SHR + E, SHR + β -estradiol.

WKY rats, tail arterial BP was significantly increased in the SHR group (97.03 ± 1.63 vs. 144.91 ± 12.1 mmHg, respectively; $P < 0.01$; Fig. 1). However, compared with the SHR group, β -estradiol significantly ameliorated the increase in tail arterial pressure in the SHR + β -estradiol (E) group (144.91 ± 12.1 vs. 110.0 ± 5.9 mmHg, respectively; $P < 0.05$; Fig. 1). There was no difference in tail arterial pressure between the SHR+E group and WKY group (110.0 ± 5.9 vs. 97.03 ± 1.63 mmHg, respectively; $P > 0.05$; Fig. 1).

β -estradiol prevents vascular remodeling and renal damage in SHRs. Hematoxylin and eosin staining results revealed that cerebral arteries of SHRs exhibited increased medial wall thickness and severe endothelium injury with increased

infiltration of inflammatory cells compared with WKYs (Fig. 2). Furthermore, hypertension also resulted in marked renal damage, demonstrated by enlarged renal tubules, atrophy of glomerular and tubular epithelial cells, interstitial expansion and accumulation of inflammatory cells in SHRs (Fig. 2). By contrast, administration of β -estradiol ameliorated hypertension-induced structural changes and inflammation in cerebral arteries and kidneys compared with the SHR group (Fig. 2).

β -estradiol suppresses hypertension-mediated inflammation by reversing the imbalance of T lymphocyte subsets and inhibiting pro-inflammatory cytokines production in SHR. To investigate whether estrogen ameliorates

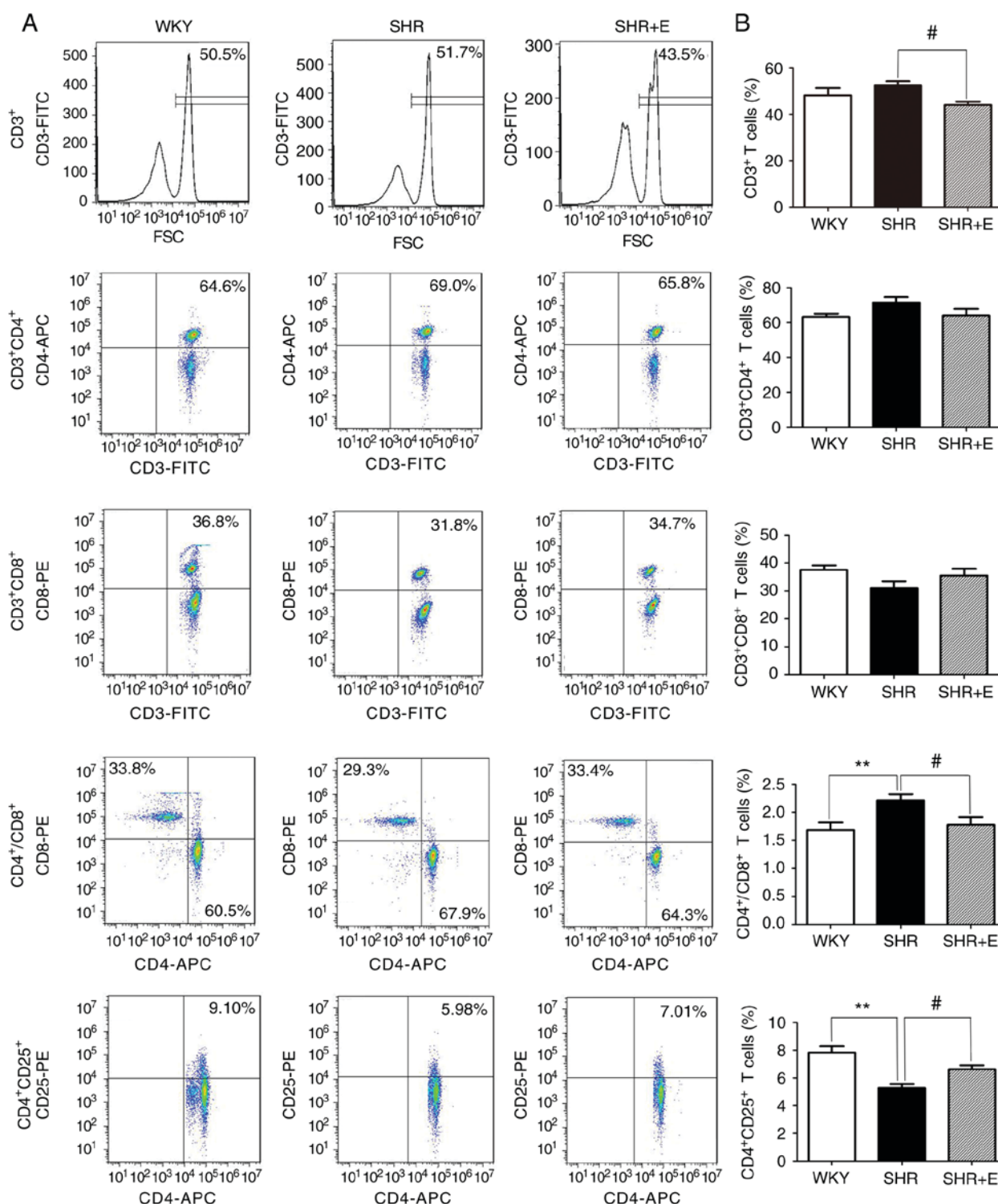


Figure 3. β -estradiol supplement reverses the changes in the percentages of various T lymphocyte subpopulations in peripheral circulation of SHR. The percentage of CD3⁺, CD3⁺CD4⁺, CD3⁺CD8⁺, CD4⁺CD25⁺ T cells was analyzed by flow cytometry. (A) Flow cytometry dot plots represent the percentages of circulating T lymphocyte subtypes in the peripheral blood of SHR and WKY rats. Decrease in the CD3⁺ T cell population and CD4⁺CD8⁺ T cell subset ratios, and increased percentage of CD4⁺CD25⁺ T cells were observed following β -estradiol treatment in SHR. (B) Bar graphs represent the percentage of various T cell subpopulations and CD4⁺CD8⁺ T cell subset ratios. All numerical data are displayed as the mean \pm standard error ($n=6$ /group). ** $P<0.01$ vs. WKY group and SHR group without any drug treatment. # $P<0.05$ SHR group without any drug treatment vs. SHR group given β -estradiol. SHR, spontaneously hypertensive rat; WKY, Wistar-Kyoto rats; CD, cluster of differentiation; SHR + E, SHR + β -estradiol; FITC, fluorescein isothiocyanate; PE, phycoerythrin; APC, allophycocyanin; FSC, forward scatter.

hypertension-mediated inflammation, the effect of β -estradiol on the percentage of lymphocyte subpopulations in peripheral blood of SHR was evaluated using flow cytometry. In the SHR group without β -estradiol supplement, there was a significant

increase in the percentage of CD4⁺CD8⁺ T cell subset ratios compared with WKY rats (WKY vs. SHR, 1.69 ± 0.13 vs. 2.28 ± 0.11 , respectively; $P<0.01$; Fig. 3B); whereas there were no statistically significant differences among CD3⁺ (WKY

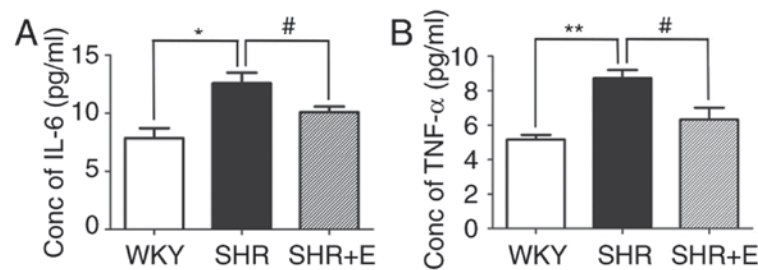


Figure 4. β -estradiol supplement attenuates hypertension induced cytokine production *in vivo*. Concentrations of (A) IL-6 and (B) TNF- α in serum of WKY, SHR and SHR received β -estradiol treatment. Data are presented as the mean \pm standard error of three independent experiments (n=6/group). * P <0.05 and ** P <0.01 vs. the WKY group and SHR group without any drug treatment. # P <0.05, SHR group without any drug treatment vs. SHR group given β -estradiol. TNF- α , tumor necrosis factor- α ; IL, interleukin; WKY, Wistar-Kyoto rats; SHR, spontaneously hypertensive rat; SHR + E, SHR + β -estradiol.

vs. SHR, 48.12 ± 3.12 vs. $52.53 \pm 1.68\%$; $P > 0.05$; Fig. 3B) and CD4⁺ (WKY vs. SHR, 64.76 ± 1.23 vs. $68.66 \pm 1.44\%$; $P > 0.05$; Fig. 3B) populations in the SHR group compared with the WKY group. Compared with the WKY group, the percentage of CD4⁺CD25⁺ T cells obtained from the peripheral blood of SHR was significantly decreased (WKY vs. SHR, 7.83 ± 0.46 vs. $5.26 \pm 0.31\%$; $P < 0.01$; Fig. 3B). However, β -estradiol treatment resulted in a significant decrease in the percentage of CD3⁺ T cells (SHR vs. SHR + E, 52.53 ± 1.68 vs. $43.96 \pm 1.26\%$; $P < 0.05$; Fig. 3B) and CD4⁺/CD8⁺ T cell subset ratios (SHR vs. SHR + E, 2.28 ± 0.11 vs. 1.78 ± 0.14 ; $P < 0.05$; Fig. 3B) compared with SHR. In particular, β -estradiol induced a significant increase in the percentage of CD4⁺CD25⁺ T lymphocytes in SHRs compared with SHR controls (SHR vs. SHR + E, 5.26 ± 0.31 vs. $6.62 \pm 0.29\%$; $P < 0.05$; Fig. 3B), which may be associated with increased Treg production stimulated by β -estradiol.

The influence of β -estradiol on TNF- α and IL-6 secretion was evaluated in serum isolated from SHRs. The analysis revealed that serum from SHRs that received β -estradiol exhibited a significantly lower level of plasma IL-6 (SHR vs. SHR + E, 12.37 ± 0.67 vs. 10.11 ± 0.42 pg/ml; $P < 0.05$; Fig. 4A) and TNF- α (SHR vs. SHR + E, 8.94 ± 0.40 vs. 6.17 ± 0.96 pg/ml; $P < 0.05$; Fig. 4B) compared with the SHR control group.

Effect of β -estradiol on the expression of Cxs in peripheral blood lymphocyte subsets of SHRs. To determine whether β -estradiol affects the expression of Cxs, conventional flow cytometry was used to evaluate total protein expression of Cx40 and Cx43 in peripheral blood lymphocyte subsets from SHRs. Consistent with the authors' previous study (12), flow cytometric analysis revealed that the percentages of CD8⁺Cx40⁺ (WKY vs. SHR, 28.14 ± 1.55 vs. $34.80 \pm 1.22\%$; $P < 0.01$), CD4⁺Cx43⁺ (WKY vs. SHR, 44.75 ± 3.02 vs. $53.52 \pm 0.97\%$; $P < 0.05$) and CD8⁺Cx43⁺ (WKY vs. SHR, 28.09 ± 1.80 vs. $38.82 \pm 1.44\%$; $P < 0.01$) double-positive peripheral blood T lymphocytes were significantly increased in SHRs compared with WKYs; whereas, there were no statistically significant differences in the percentages of Cx40 and CD4 double-positive T lymphocytes between the SHR and WKY groups (Fig. 5A and B). β -estradiol supplementation exhibited an inhibitory effect on the abundance of Cx40 and Cx43 in CD4⁺ or CD8⁺ cells. β -estradiol significantly decreased the amount of CD8⁺Cx40⁺ (SHR vs. SHR + E,

34.80 ± 1.22 vs. $30.53 \pm 1.24\%$; $P < 0.05$), CD4⁺Cx43⁺ (SHR vs. SHR + E, 53.52 ± 0.97 vs. $49.65 \pm 0.87\%$; $P < 0.05$; Fig. 5A and B) and CD8⁺Cx43⁺ (SHR vs. SHR + E, 38.82 ± 1.44 vs. $33.3 \pm 1.89\%$; $P < 0.05$) double-positive peripheral blood T lymphocytes compared with the SHR controls (Fig. 5A and B).

Pretreatment with β -estradiol inhibits the release of pro-inflammatory cytokines in T-cell mitogen-stimulated peripheral blood lymphocytes. To further assess whether β -estradiol has inhibitory effects on the release of pro-inflammatory cytokines, the levels of pro-inflammatory cytokines released by peripheral blood T lymphocytes were determined using the culture supernatant of peripheral blood lymphocytes *in vitro*. IL-6 (Control vs. Con A, 38.68 ± 1.53 vs. 44.92 ± 1.90 pg/ml; $P < 0.05$; Fig. 6A) and TNF- α (Control vs. Con A, 16.39 ± 0.80 vs. 25.28 ± 0.99 pg/ml, $P < 0.05$; Fig. 6B) concentrations were significantly enhanced by Con A compared with the untreated controls. However, pre-treatment with β -estradiol (10 nM) significantly attenuated the secretion of IL-6 (Con A vs. Con A + E, 44.92 ± 1.90 vs. 37.87 ± 1.36 pg/ml; $P < 0.05$; Fig. 6A) and TNF- α (Con A vs. Con A + E, 25.28 ± 0.99 vs. 21.72 ± 0.49 pg/ml; $P < 0.05$; Fig. 6B) in peripheral blood T lymphocytes. Overall, the data demonstrated that β -estradiol treatment has potent inhibitory effects on T lymphocyte-induced inflammatory responses.

β -estradiol suppresses the function of gap junctions between peripheral blood lymphocytes in the presence of T cell mitogenic stimuli. To confirm whether Cxs-based gap junctions are involved in inhibitory effects of β -estradiol on hypertension-mediated inflammation, a functional *in vitro* assay based on the transfer of a gap junction permeant dye was used to evaluate the functional alteration of Cx-mediated gap junctions in peripheral blood lymphocytes in the presence of β -estradiol or/and Con A. Dye transfer between lymphocytes was evaluated by flow cytometry. Following Con A treatment, there was a significant increase in calcein AM dye transfer between cocultured cells, as demonstrated by an increased percentage of DiIC₁₈-calcein double-positive cells compared with the unstimulated control (Control vs. Con A, 4.41 ± 0.37 vs. $12.1 \pm 0.76\%$, $P < 0.01$; Fig. 7A and B). Treatment with β -estradiol significantly suppressed the Con A-induced increase in dye transfer between lymphocytes (Con A vs. Con A + E, 12.1 ± 0.76 vs. $7.69 \pm 1.13\%$, $P < 0.05$; Fig. 7A and B).

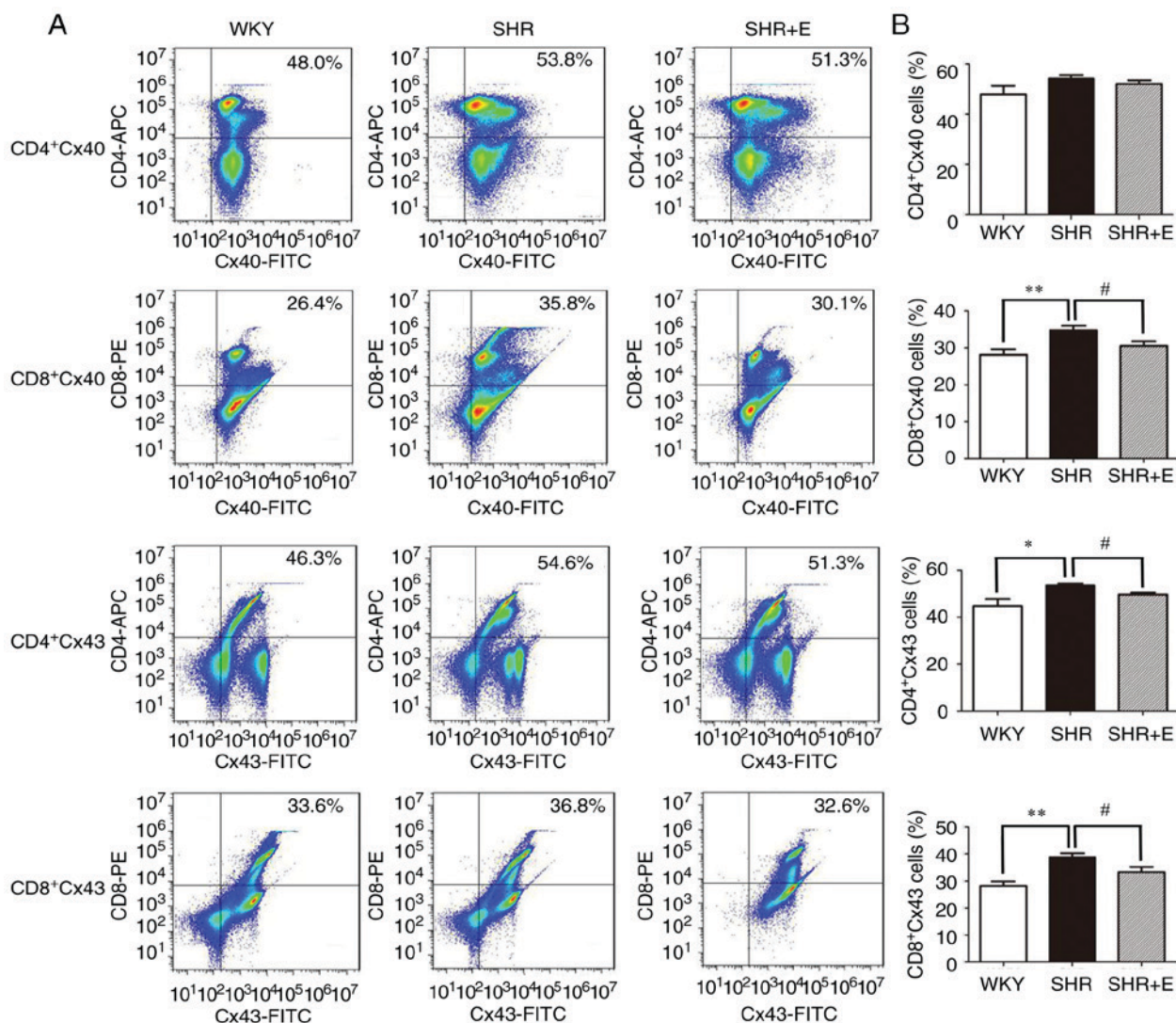


Figure 5. β -estradiol supplement reduces the percentages of Cx40- and Cx43-expressing CD4⁺ and CD8⁺ T cells in SHR. (A) Representative dot plot and (B) collated data presenting percentages of Cx40- and Cx43-positive CD4⁺ and CD8⁺ T cells in each group. Data are presented as the mean \pm standard error of three independent experiments (n=6/group). *P<0.05 and **P<0.01, WKY group vs. SHR group without any drug treatment; #P<0.05, SHR group without any drug treatment vs. SHR group with β -estradiol treatment. Cx, connexin; SHR, spontaneously hypertensive rat; WKY, Wistar-Kyoto rats; SHR + E, SHR + β -estradiol; CD, cluster of differentiation; FITC, fluorescein isothiocyanate; PE, phycoerythrin; APC, allophycocyanin.

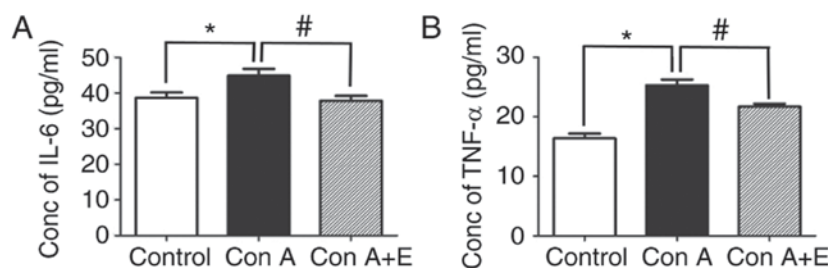


Figure 6. β -estradiol pre-treatment decreased Con A-induced secretion of pro-inflammatory cytokines in the culture supernatant of peripheral blood lymphocytes stimulated with Con A. Contents of (A) IL-6 and (B) TNF- α in the culture supernatant of unstimulated, Con A stimulated and β -estradiol treated peripheral blood lymphocytes. Data are presented as the mean \pm standard error (n=5). *P<0.05, control group vs. the Con A-stimulated group; #P<0.05, Con A-stimulated group vs. the β -estradiol treated group. Each figure is representative of three independent experiments. Con A, concanavalin A; Con A + E, concanavalin A + β -estradiol; IL, interleukin; TNF, tumor necrosis factor.

Pretreatment with β -estradiol inhibits the expression of Cx40 and Cx43 in Con A stimulated peripheral blood lymphocytes. The effect of β -estradiol on the expression of Cx40 and Cx43

in Con A-stimulated peripheral blood lymphocytes was assessed using confocal microscopy and immunoblotting. The protein levels of Cx40 and Cx43 were significantly enhanced

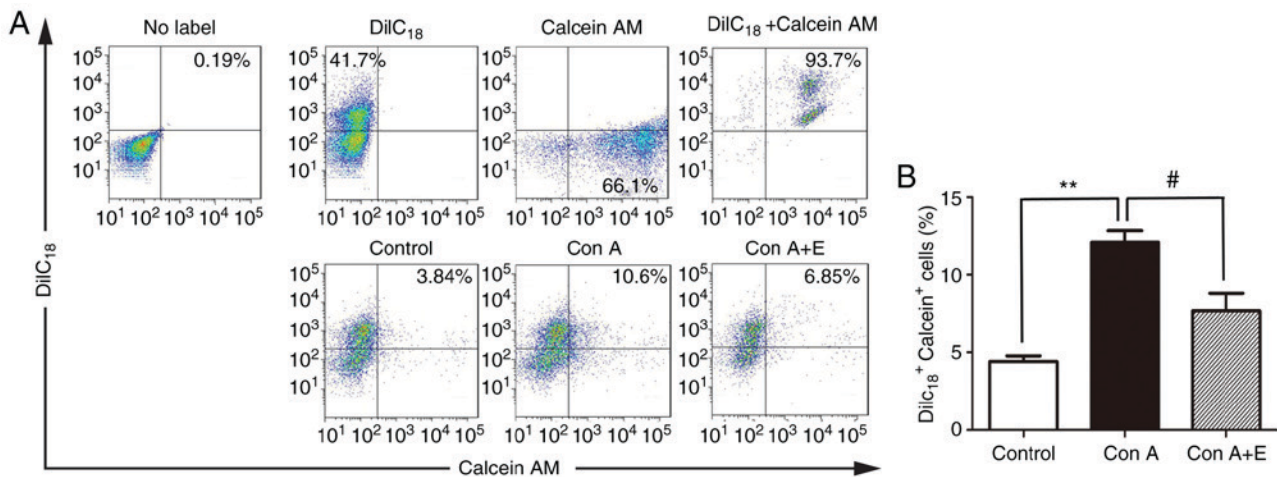


Figure 7. β -estradiol supplement suppresses the function of gap junctions in Con A stimulated peripheral blood lymphocytes. (A) Control experiments of DiIC₁₈ or calcein AM single-stained cells and DiIC₁₈-calcein double-stained cells were performed in parallel; DiIC₁₈⁺-calcein⁺-positive fluorescent cells expressed as a represent percentage of the total number of peripheral blood lymphocytes. (B) Mean percentages of DiIC₁₈⁺-calcein⁺-double-positive cells of three independent experiments \pm standard error from five samples/group. ** $P < 0.01$, control group vs. Con A-stimulated group; # $P < 0.05$, Con A-stimulated group vs. E-treated group. Con A, concanavalin A; calcein AM, calcein acetoxymethyl ester; E, β -estradiol.

in Con A-stimulated peripheral blood lymphocytes, as demonstrated by immunofluorescence ($P < 0.01$ for Cx40; $P < 0.05$ for Cx43; Fig. 8A and B) and western blot analysis ($P < 0.01$ for Cx40; $P < 0.05$ for Cx43; Fig. 9A and B); by contrast, pretreatment with β -estradiol significantly reduced the Con A-induced increase Cx40 and Cx43 expression in peripheral blood lymphocytes as demonstrated by immunofluorescence ($P < 0.05$ for Cx40 and Cx43; Fig. 8A and B) and western blot analysis ($P < 0.05$ for Cx40 and Cx43; Fig. 9A and B).

Discussion

In the present study, the effects of β -estradiol on the hypertension-mediated adaptive immune response were investigated in male SHR. The primary findings of the present study were that β -estradiol significantly prevented inflammation and target organ damage (kidneys and arteries) in male SHR. Furthermore, while hypertension appears to promote imbalances in the adaptive immune response through increased expression of Cxs (Cx40 and Cx43), which have previously been demonstrated to be pro-inflammatory (10-16), β -estradiol appeared to alleviate hypertension-mediated imbalances of the adaptive immune response and T cell mitogenic stimuli-induced adaptive immune response, at least in part, by suppressing the expression of Cxs or the function of Cx gap junctions. β -estradiol inhibited the release of pro-inflammatory cytokines induced by spontaneous hypertension or T cell activation. Therefore, the findings of this study support the dual anti-hypertensive and anti-inflammatory role of estrogen and extends the understanding of the cellular mechanisms underlying the regulatory role of estrogen in BP and hypertension-mediated inflammation.

It is well established that hypertension and hypertension-induced organ damage are associated with excessive inflammatory responses in experimental models of hypertension and hypertensive patients (17,29). Male hypertensive animal models (angiotensin II, deoxycorticosterone acetate-salt and SHR) and hypertensive patients exhibit a marked increase

in the number of circulating T lymphocytes, vascular and renal infiltration of CD4⁺ and CD8⁺ T cells, and the levels of pro-inflammatory cytokines, including IL-6 and TNF- α , compared with normotensive controls (3,30,31). The present data also demonstrated that hypertension-mediated inflammation and hypertension-induced vascular and renal infiltration of inflammatory cells is associated with alterations in the number of effector T lymphocytes (CD4⁺ and CD8⁺ T cells) and Tregs, and the production of pro-inflammatory cytokines in the peripheral blood of SHR. Although it is clear that T lymphocytes have a key role in hypertension development, the precise mechanism leading to activation and proliferation of T lymphocytes in hypertension remains poorly defined. Notably, other data from the authors' group and the current findings demonstrate the importance of Cx-mediated gap junctions between peripheral blood T lymphocytes in hypertension-mediated pro-inflammatory responses (10-13); increased Cx43 expression and enhanced Cx43-mediated gap junctional communication, in particular, is important for pro-inflammatory responses driven by T lymphocytes and the production of T cell-induced cytokines under different pro-inflammatory stimuli (10,11). In the present study, Cx40 and Cx43 expression was increased in peripheral blood lymphocytes from SHR, and there was a strong association between the levels of pro-inflammatory cytokines (IL-6 and TNF- α) in the serum/culture supernatant and the expression levels of Cx40 or Cx43 in peripheral blood T lymphocytes from SHR/Con A-stimulated cells. Furthermore, the present results also demonstrated that pro-inflammatory stimuli (Con A) enhanced gap junctional communication between peripheral blood lymphocytes from WKY rats. The data further demonstrated that gap junctions are involved in the pathological mechanisms of hypertension-mediated inflammation and interventions targeted to Cx43-mediated gap junctions in effector T lymphocytes may represent a novel therapeutic strategy for the treatment of hypertension.

While T cell-targeted immunosuppressant agents and inhibition of pro-inflammatory cytokines prevent or ameliorate

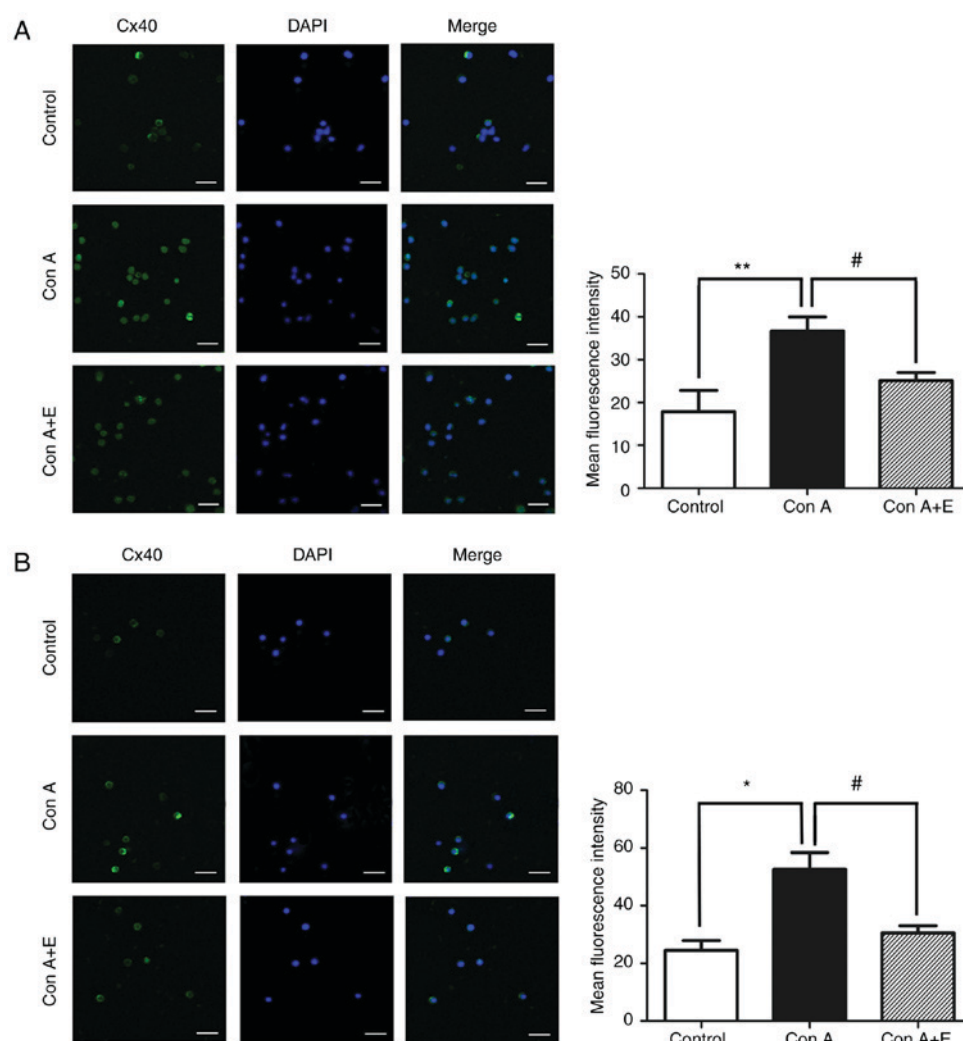


Figure 8. β -estradiol pre-treatment decreased Cx40/Cx43 expression in peripheral blood lymphocytes stimulated with Con A. Representative immunofluorescence images of (A) Cx40 and (B) Cx43 (magnification, $\times 630$; scale bar, $16 \mu\text{m}$) to determine their localization and expression in the plasma membrane and cytosol. These images presenting the changes of Cx40 and Cx43 expression when Con A stimulated peripheral blood lymphocytes were pretreated with β -estradiol. Bar graphs presenting densitometric analysis of mean immunofluorescent intensity of Cx40 and Cx43 in various groups. Each figure is representative of three independent experiments. Each column represents the mean \pm standard error from five samples/group. * $P < 0.05$ or ** $P < 0.01$, control group vs. Con A-stimulated group; # $P < 0.05$, Con A-stimulated group vs. E-treated group. Cx, connexin; Con A, concanavalin A; DAPI, 4',6-diamidino-2-phenylindole; E, β -estradiol.

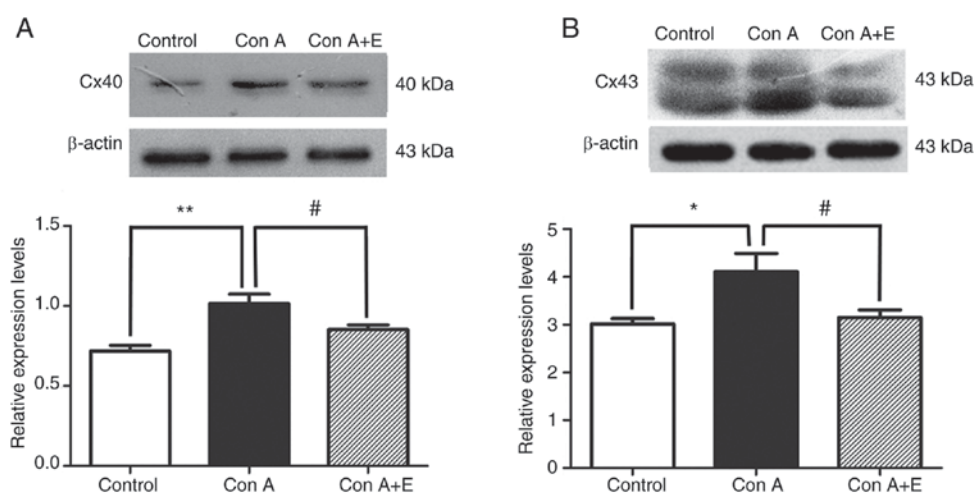


Figure 9. β -estradiol pre-treatment inhibits Con A-induced protein expression of Cx40 and Cx43 in peripheral blood lymphocytes. The immunoblot analysis of (A) Cx40 and (B) Cx43 in peripheral blood lymphocytes treated with Con A and Con A + β -estradiol. The bar graph presents the densitometric analysis of relative expression of (A) Cx40 and (B) Cx43 normalized to β -actin level. Results are presented as the mean \pm standard error of five samples/group. * $P < 0.05$ and ** $P < 0.01$, control group vs. Con A-stimulated group; # $P < 0.05$, Con A-stimulated group vs. β -estradiol treated group. Each figure is representative of three independent experiments. Con A, concanavalin A; Cx, connexin.

BP elevation and hypertension-mediated inflammation in experimental models of hypertension and in hypertensive patients (1,3,9,17,18,21), these immunosuppressive drugs may result in unwanted side effects in patients and animals with hypertension (21). In females, estrogen has been revealed to have a central role in preventing the development of hypertension and suppressing inflammation-mediated diseases (32,33), which has been well established in studies of women with ovarian hormone deficiency and male animal models of hypertension (20-24,33,34). Estrogen has also been reported to provide protective effects against hypertension-induced vascular remodeling and renal damage by reducing infiltration of leukocytes or effector T lymphocytes (total T cells, CD4⁺ or Th17 cells) into organs (brain, heart, kidney and vasculature), and enhancing the number of anti-inflammatory Tregs in renal tissues (21,31,35) in female hypertension models (SHR and female Rag-1^{-/-} mice infused with angiotensin II following adoptive transfer of CD3⁺ T cells from wild-type male mice). Similarly, estrogen replacement (17 β -estradiol) was also demonstrated to reduce leukocyte/macrophage infiltration in cardiac and vascular tissues from ovariectomized hypertensive rats, aldosterone salt-treated rats and ovariectomized rats subjected to balloon injury of the right carotid artery (26,36). In the current study it was also observed that β -estradiol administration to male SHRs reduced BP and prevented vascular remodeling (arterial wall thickening), and inhibited leukocyte infiltration in the vascular wall and kidneys. The results demonstrated that estrogen has a protective role in the regulation of BP and target organ damage. Another important finding from the present study was that long term treatment with β -estradiol significantly attenuated the imbalance between effector and regulatory T cell subsets in SHRs, potentially by reducing serum levels of TNF- α and IL-6, and the CD4/CD8 ratio, and enhancing the number of Tregs; whereas β -estradiol exhibited no effect on the proportion of CD4⁺ and CD8⁺ T cells in the peripheral blood of SHRs. The increasing percentage of Tregs in β -estradiol-treated SHRs may be associated with β -estradiol-stimulated conversion of CD4⁺ T cells into Tregs (21) and decreased pro-inflammatory cytokines levels imply that β -estradiol attenuates the hypertension-mediated pro-inflammatory response in the circulation.

Furthermore, a previous study indicated that the menopause or estrogen deficiency in monocytes cultured *ex vivo* is associated with increased levels of pro-inflammatory cytokines produced by monocytes (IL-1, IL-6 and TNF- α) (22); while higher physiological or supraphysiological levels of estrogen attenuate aberrant expression or production of TH1-driven pro-inflammatory cytokines (IL-6, TNF- α , interferon- γ and monocyte chemoattractant protein-1) in estrogen-deficient animals (32), and in various inflammatory and cardiovascular diseases, including stroke (37), balloon injury of carotid arteries (36) and hypertension (34). To further determine the anti-inflammatory role of estrogen against inflammatory stimuli-induced secretion of pro-inflammatory cytokines, the effect of β -estradiol pretreatment on IL-6 and TNF- α secretion in PBMCs following exposure to T cell mitogenic stimulus (Con A) was investigated to determine whether β -estradiol suppresses secretion of the two cytokines. Pretreatment with β -estradiol significantly reduced Con A-stimulated secretion of IL-6 and TNF- α in peripheral

blood lymphocytes from WKY rats, which supports the findings from the SHR serum. These results also confirmed an association between estrogen signaling and the production of pro-inflammatory cytokines.

Several other studies have reported that estrogen inhibits inflammatory infiltration via an estrogen receptor-dependent mechanism (28,33,38-40). Subsequently the mechanisms by which estrogen suppresses hypertension-mediated inflammation and secretion of pro-inflammatory cytokines, and whether this effect is associated with the expression of Cxs and the function of Cx-mediated gap junctions between peripheral blood lymphocytes was determined. β -estradiol treatment markedly suppressed the expression of Cxs in peripheral blood lymphocytes from SHRs and in Con A-stimulated peripheral blood lymphocytes *in vitro*, and reduced Cx-mediated gap junctional communication. The role of Cx gap junctions in hypertension-mediated inflammatory response was preliminarily demonstrated in the authors' previous studies (10-13), whereby blockade of Cx43-mediated gap junctions between T lymphocytes led to reduced expression and production of various pro-inflammatory cytokines by peripheral blood lymphocytes in SHRs and hypertensive patients (10,11). Therefore, on the basis of these observations, it is logical to hypothesize that β -estradiol supplementation could affect the peripheral blood lymphocyte subsets in SHRs and suppress production of pro-inflammatory cytokines by inhibiting the expression and function of Cxs in peripheral blood lymphocytes.

The current study has certain limitations. The levels of estrogen in peripheral blood/culture supernatant were not detected, although estrogen effectively ameliorated BP elevation, hypertension-mediated inflammation and hypertension-induced organ damage in SHRs, and reduced Con A-induced secretion of pro-inflammatory cytokines *in vitro*. Additionally, although estrogen receptors α and β are expressed on the majority of immune cells (41) and estrogen receptor α signaling in T lymphocytes is required for the protective effect of estrogen via estradiol-mediated inhibition of Th1 and Th17 cell differentiation (33), whether estrogen receptors α and β are expressed in peripheral blood lymphocytes from different experimental hypertensive animals and hypertensive patients is unknown, and their expression profile in hypertensive models following estrogen treatment has not been established. Consequently, future studies are required to determine whether estrogen inhibits hypertension-mediated inflammation in different hypertensive models and hypertensive patients via estrogen receptor-dependent or independent signaling pathways. Finally, the detailed anti-inflammatory mechanism of estrogen via regulation of Cxs-based channel functions (gap junction channels or hemi-channels) during hypertension or pro-inflammatory stimuli-mediated inflammatory responses was not investigated, although Cx-mediated hemi-channels are known to be activated in inflammatory/pathological conditions. Further studies will also be required to determine the role of gap junctions or hemi-channels in the protective effects of estrogen in hypertension-mediated inflammation; this may involve using gap junction- and hemi-channel-specific mimetic peptides (42) in combination with lentivirus-mediated RNA interference knockdown of Cx43.

In conclusion, the results of the present study indicate that β -estradiol inhibits BP elevation and target organ damage in male SHR. In addition, the results suggest that β -estradiol contributes to the inhibition of hypertension- or pro-inflammatory stimuli-mediated inflammatory responses and production of pro-inflammatory cytokines. These effects may be mediated, at least in part, by suppressing the function of Cx-based gap junctions and Cx protein expression (Cx40 and Cx43). These results provide novel insight into the molecular mechanisms by which estrogen can reduce hypertension. The present results and previous studies indicate that Cxs and Cx-mediated gap junctions are critical contributing factors in hypertension-mediated inflammation and may be useful as targets for the treatment of hypertension and other cardiovascular diseases.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

KTM, XZL and YYZ conceived and designed the experiments. XN performed the experiments. LZ, XM, LYS, LL and JQS analyzed the data. LZ wrote the manuscript. All authors read and approved the final manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study does not report on studies involving human participants, human data or human tissue. The protocol of the study was approved by Institutional Animal Care and Use Committees (permit no. A2016-047-03) of the Medical College of Shihezi University, and all animal handling and experimental procedures were performed in accordance with guidelines for the Care and Use of Laboratory Animals published by the US National Institutes of Health (Public Health Service Policy on Humane Care and Use of Animals, DHEW Publication no. 96-01, PHS Policy revised in 2002).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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