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Which comes first: Renal inflammation or oxidative stress in spontaneously hypertensive rats?

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Abstract
The present study was undertaken to identify whether inflammation or oxidative stress is the primary abnormality in the kidney in spontaneously hypertensive rats (SHR). Renal inflammation and oxidative stress were evaluated in 2- and 3-week-old prehypertensive SHR and age-matched genetically normotensive control Wistar-Kyoto (WKY) rats. Blood pressure was similar in WKY and SHR rats at 2 and 3 weeks, of age. Renal inflammation (macrophage and nuclear factor-κB) was elevated in SHR at 3 weeks, but not at 2 weeks, of age compared with age-matched WKY rats. Renal oxidative stress (nitrotyrosine, 8-hydroxy-2′-deoxyguanosine and p47phox) was also clearly elevated in 3-week-old SHR compared with age-matched WKY rats. Additionally, NADPH oxidase subunit p47phox was found elevated in 2-week-old SHR compared to age-matched WKY rats. Moreover, antioxidant (*N*-acetyl-L-cysteine and Tempol) treatment reduced renal inflammation in prehypertensive SHR. We therefore conclude that the oxidative stress appears before inflammation as a primary abnormality in the kidney in prehypertensive SHR.

Keywords: *Oxidative stress, inflammation, SHR, p47phox, nitrotyrosine, NF-κBp65*

Introduction

Spontaneously hypertensive rats (SHR) are commonly used as a model of human essential hypertension (HTN). SHR remain normotensive until 4–5 weeks of age, and then the blood pressure (BP) gradually increases in these animals and they become fully hypertensive by around 10–12 weeks of age [1]. Recent studies have convincingly shown that the HTN in different animal models, including SHR, is tightly associated with oxidative stress and inflammation in the kidney [2–8]. Antioxidants have been found to reduce renal inflammation and BP as well as renal oxidative stress in adult SHR [2,7]. On the other hand, anti-inflammatory agents have been shown to reduce oxidative stress and BP along with reduction of renal inflammation in SHR [3,6]. Thus, inflammation and oxidative stress in the kidney and systemic HTN are interdependent in adult hypertensive SHR.

Recently it has been demonstrated that the inflammation and oxidative stress develop in the kidney of SHR at or before the age of 4 weeks, the age at which the rats are still normotensive [5,9]. This evidence, therefore, strongly supports the hypothesis that the renal inflammation and/or oxidative stress may be causally linked to the development of systemic HTN in SHR. However, it is still unknown whether renal inflammation is the cause or the effect of renal oxidative stress in SHR. Considering the clinical relevance of identification of primary cause in the pathogenesis of HTN, the present study was designed to identify the primary abnormality between renal inflammation and oxidative stress in SHR. We studied oxidative stress and inflammation in the

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kidney of 2- and 3-week-old prehypertensive SHR and age-matched genetic control Wistar-Kyoto (WKY) rats. On the basis of the observational findings, we further proceeded with an interventional approach in which we treated 2-week-old SHR with antioxidants, *N*-acetyl-L-cysteine (NAC) and Tempol, for 1 week. Our findings indicate that the oxidative stress appears before inflammation as a primary abnormality in the kidney in prehypertensive SHR.

Materials and methods

Animals and experimental protocol

The protocol for this study complied with the guidelines established by the Brazilian College of Animal Experimentation (COBEA) and was approved by the Institutional Ethical Committee. All reagents were purchased from Sigma, St Louis, MO, USA, unless stated otherwise. The SHR ($n = 26$) and their genetically normotensive control WKY ($n = 24$) rats derived from animals supplied by Taconic (Germantown, NY, USA) and bred in our animal facility were used in this study. We studied BP, and renal inflammation and oxidative stress in male SHR and WKY rats at two different age points: (i) 2-week group, consisting of SHR ($n = 12$) and WKY ($n = 11$) rats that were 2 weeks of age (14–15 days); and (ii) 3-week group, consisting of SHR ($n = 14$) and WKY ($n = 13$) rats that were 3 weeks of age (20–22 days). Rats were sacrificed by overdose of pentobarbital (Hypnol®), and both of the kidneys were removed immediately, carefully wiped on cotton gauze, decapsulated and cut longitudinally into two halves. One half was fixed immersing in a solution of Methacarn [10] and subsequently embedded in paraffin. A small part of the cortical tissue was homogenized in homogenization buffer. The remaining cortical tissue was frozen in liquid nitrogen for subsequent collection of nuclear extract and determination of reduced form of glutathione (GSH).

A separate group of 2-week-old 10 SHR rats were used for antioxidant intervention study. Five of them were treated with thiol-based antioxidant NAC (Calbiochem, 200 mg/kg/day, intraperitoneal, in the morning) and superoxide dismutase mimetic Tempol (4-hydroxy-2,2,6,6,-tetramethylpiperidine-1-oxyl) (Calbiochem, 250 mg/kg/day, intraperitoneal, in the evening). Another five rats were treated with the vehicle normal saline as control. After 1-week of treatment, that is, at the age of 3-week, the rats were sacrificed and the kidney tissues were processed as described above.

Blood pressure determinations

Systolic blood pressure was obtained by tail-cuff plethysmography (three to five determinations per rat) in unanaesthetized rats using an MK III physiograph (Narco Bio-System, Houston, TX, USA). A small cuff suitable for measuring blood pressure in 2–3-week-old rats was validated in our laboratory and used in the present study. The rats were habituated to the procedure before taking the blood pressure readings.

Preparation of renal cortical extract

Renal cortical extract was prepared in tissue homogenization buffer (30 mM Tris–HCl, pH 7.5, 10 mM EGTA, 5 mM EDTA, 1 mM DTT and 250 mM sucrose) supplemented with a cocktail of protease inhibitors as described previously [11].

Preparation of nuclear extract

Nuclear extract of the kidney cortex was prepared as described previously [12] with few modifications. Snap-frozen kidney cortices were pulverized in liquid nitrogen and homogenized in homogenization buffer A (250 mM sucrose, 10 mM Hepes, pH 7.6, 25 mM KCl, 1 mM EDTA, 10% glycerol, 0.1 mM PMSF and 2.5 μg/ml aprotinin) with a Dounce homogenizer. The homogenate was layered over buffer B (1 M sucrose, 10 mMHepes, pH 7.6, 25 mM KCl, 1 mM EDTA, 10% glycerol, 0.1 mM PMSF and 2.5 μg/ml aprotinin) and centrifuged at 3900*g* for 10 min at 4°C. The pellet was resuspended in buffer A/glycerol (9:1,w/w) and layered over buffer B/glycerol (9:1,w/w). The gradient was centrifuged at 48,000*g* for 30 min at 4°C. The semi-purified nuclear pellet was resuspended in nuclear extraction buffer (10 mM Hepes, pH 7.6, 400 mM KCl, 3 mM $MgCl_2$, 0.1 mM EDTA, 10% glycerol, 1 mM DTT, 0.1 mM PMSF). Nuclear proteins were then extracted on ice for 30 min, and the particulate material was removed by centrifugation at 13,000*g* for 10 min at 4°C. An aliquot of 10 μl supernatant containing nuclear extract was collected for measuring total protein concentration (Bradford) [13], and the remaining supernatant was mixed in Laemmli sample buffer [14], heated at 100°C for 5 min, and stored at −80°C.

Immunohistochemistry

To detect macrophage infiltration, methacarn-fixed paraffin-embedded renal tissue sections (4 μm) were dewaxed and rehydrated. After microwave exposure and blocking with nonfat milk, slides were incubated with a 1:50 dilution of monoclonal mouse anti-rat ED1 antibody (Serotec, Oxford, UK) followed by alkaline phosphatase labeled polymer (Dako EnVision system, DAKO corporation, Carpinteria, CA, USA) conjugated with anti-mouse antibody, developed with fast red (Dako EnVision system, DAKO) and counterstained with hematoxylin. To detect oxidative stress-induced DNA base

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modification, immunohistochemistry was done for 8-hydroxy-2′-deoxyguanosine (8-OHdG, a DNA base-modified product) in methacarn-fixed paraffin-embedded renal tissue sections. After microwave exposure and blocking of endogenous peroxidase with 3% H_2O_2, slides were incubated with a 1:50 dilution of a mouse monoclonal anti-8-OHdG antibody (N45.1; Japan Institute for the Control of Aging, Japan), and subsequently a 1:200 dilution of a biotinylated secondary anti-mouse IgG antibody (Vector, Burlingame, CA, USA). After incubation with avidin–biotin complex (ABC) reagent (Dako, Glostrup, Denmark), slides were developed in diaminobenzidine tetrahydrochloride (DAB) and counterstained with hematoxylin. For negative controls, staining was performed omitting the primary antibody or by using an irrelevant immunoglobulin. Tubulointerstitial cells containing 8-OHdG positive nuclei were counted in 50 sequential high power microscopic fields (× 400), and three to five sections were evaluated for each animal.

Determination of reduced glutathione (GSH) concentration

Renal cortical GSH level was measured by the method of Beutler et al. [15] with few modifications. A small piece of frozen kidney cortex was weighed and was directly homogenized in cold 10% trichloroacetic acid on ice. Homogenate was centrifuged at 3000 rpm for 15 min at 4°C. The supernatant was reacted with 0.3 M phosphate buffer and 0.04% 5,5′dithio-bis 2-nitrobenzoic acid (DTNB). Absorbance was read at 412 nm and the GSH concentration was expressed as micromole GSH per gram frozen tissue. GSH (reduced form) was used as an external standard for preparation of a standard curve.

Western blotting

Renal cortical homogenate was used for quantification of nitrotyrosine, p47phox and Nox4 (homologue of NADPH oxidase subunit gp91phox), and renal cortical nuclear extract was used for quantification of nuclear factor-κB (NF-κB) p65 subunit. Fifty micrograms of cortical or nuclear protein was separated on 10% SDS-polyacrylamide gel and electrophoretically transferred to nitrocellulose membrane using Mini-Protean II Dual Slab Cell apparatus (Bio-Rad Laboratories, Hercules, CA, USA). After blocking nonspecific binding, the membranes were incubated with a mouse monoclonal anti-nitrotyrosine antibody (1:2000; clone 1A6, Upstate, Lake Placid, NY, USA), a mouse monoclonal anti-p47phox antibody (1:500, BD Transduction Laboratories, BD Biosciences Pharmingen, NJ, USA), a rabbit polyclonal anti-Nox4 antibody (1:2500; a gift of Dr Karen Block, University of Texas Health Science Center at San Antonio) or a rabbit polyclonal anti-NF-κB-p65 antibody (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The membranes were subsequently incubated with horseradish peroxidase-conjugated appropriate secondary antibodies (Santa Cruz). Immunoreactive bands were made visible using the enhanced chemiluminescence method (Super Signal CL-HRP Substrate System; Pierce, Rockford, IL, USA). To verify the uniformity of protein load and transfer efficiency across the test samples, membranes were reprobed for actin (goat polyclonal anti-actin antibody, 1:1000, Santa Cruz), or stained with Ponceau S stain in case of nuclear proteins. Exposed films were scanned with a laser densitometer (Bio-Rad) and were analysed quantitatively with Multi-Analyst Macintosh Software for Image Analysis Systems (Bio-Rad).

Statistical analysis

Statistical significance was assessed by unpaired comparisons using Student's *t*-test. The number of 8-OHdG positive cells in immunohistochemistry was not normally distributed; nonparametric Mann–Whitney *U*-test was used in this case. All data are expressed as means ± SD except the number of 8-OHdG, which is expressed as median (range). Statistical significance was set at $p < 0.05$. All analyses were performed using statistical software StatView (SAS Institute Inc., Cary, NC, USA).

Results

In the present study, we did age-matched comparison between WKY and SHR rats, and abnormality of any parameter of SHR has been considered when it significantly differed from that of age-matched WKY rats. As we did not perfuse the rats/kidneys, we could not completely exclude the possibility of blood contamination of our samples. However, we processed tissues from WKY and SHR groups exactly the same way, and therefore the effect of possible blood contamination should be equal in both groups resulting in no/negligible effect on the overall result.

Body weight and systolic blood pressure

As we observed in previous studies, [11,16] the SHR used in this study were also smaller than the age-matched WKY rats (body weight: 2-week group, 32 ± 6 g vs. 23 ± 4 g; 3-week group, 67 ± 8 vs. 36 ± 6; WKY vs. SHR, respectively; $p < 0.001$ in both age groups). However, systolic BP (in mm Hg) was not different between WKY and SHR rats at 2 weeks (WKY: 81 ± 11 vs. SHR: 80 ± 13) and at 3 weeks (WKY: 97 ± 10 vs. SHR: 103 ± 14) of age (Figure 1).

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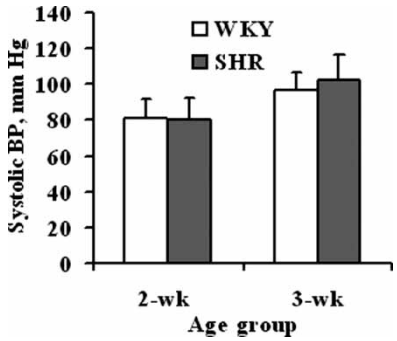

Figure 1. Systolic blood pressure in WKY and SHR rats during early development. Absolute values of blood pressure (means ± SD) in mm Hg are given in the text. Number of rats: 2-week group–12 SHR and 11 WKY rats; and 3-week group–14 SHR and 13 WKY rats.

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Inflammatory events

Macrophage infiltration. Inflammatory process in the kidney cortex was evaluated by counting macrophage (ED1-positive cell) infiltration in the tubulointerstitial area. Tubulointerstitial macrophage infiltration was similar in WKY (21.3 ± 4.7/HPF) and SHR (24.5 ± 4.3/HPF) rats at 2 weeks of age ($n = 5$ in each group, Figure 2). However, macrophage infiltration in the tubulointerstitial area was found elevated in 3-week-old SHR compared to age-matched WKY rats (WKY: 18.9 ± 1.2/HPF vs. SHR: 24.3 ± 1.6/HPF, $p = 0.0002$, $n = 5$ in each group, Figure 2).

NF-κB activation. To identify the underlying mechanism of the inflammatory process, we studied activation status of the pro-inflammatory transcription factor NF-κB by quantifying the intra-nuclear fraction of the p65 subunit. Activated NF-κB regulates the expression of adhesion molecules and chemokines, and thereby regulates the infiltration of inflammatory cells in the tissue [17]. Western blot analysis of the renal cortical nuclear extract showed that the intra-nuclear p65 (expressed in densitometric unit) was lower in SHR than in WKY rats at 2 weeks of age (WKY: 1.0 ± 0.10 vs. SHR: 0.43 ± 0.11, $p = 0.003$) (Figure 3). However, the expression and/or nuclear translocation of the p65 subunit was found elevated by 2.8 fold in 3-week-old SHR compared to age-matched WKY rats (WKY: 1.0 ± 0.39 vs. SHR: 2.81 ± 0.48, $p = 0.001$) (Figure 3). Probably, the base-line inflammatory gene expression pattern was not affected by the lower levels of intra-nuclear NF-κBp65 in SHR at 2 weeks of age, for which we observed similar levels of tubulointerstitial macrophage infiltration between WKY and SHR rats at the 2-week time point.

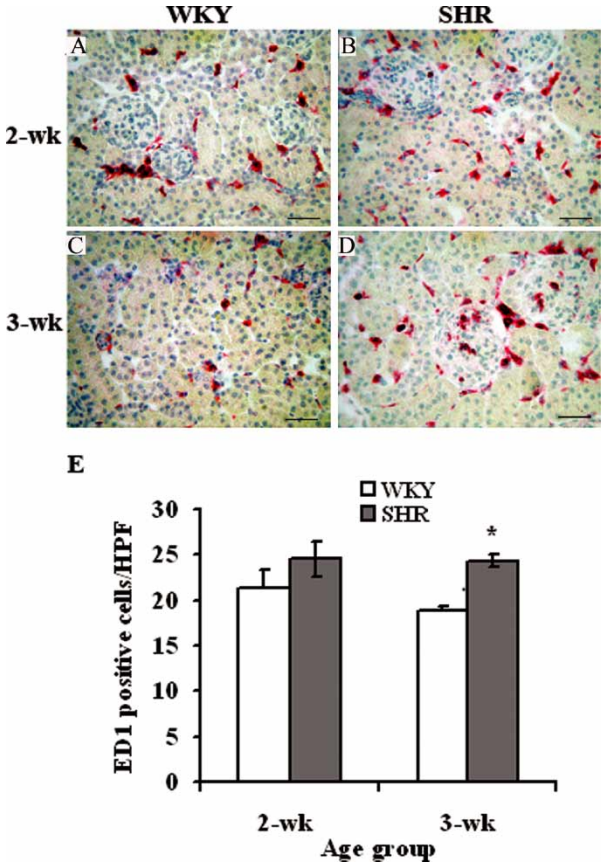

Figure 2. Immunohistochemical detection of macrophage (ED1-positive cell) infiltration in the kidney of WKY and SHR at 2 (A, B) and 3 weeks (C, D) of age. Positive staining was observed as bright-red granular staining. Tubulointerstitial macrophage infiltration was evaluated by counting ED1-positive cells in 20 cortical high power fields and expressed as positive cells per high power field (E). Three sections from each animal were evaluated. Bars are representing means ± SD; $n = 5$ in each group. HPF, high power field; $*p = 0.0002$ vs. age-matched WKY rats. Sections were counterstained with hematoxylin (original magnification × 400). Scale bar = 50 μm.

Oxidative stress

Renal cortical nitrotyrosine. Oxidative and nitrosative stress-induced protein modification (nitration of tyrosine residues leading to formation of nitrotyrosine) was assessed in the renal cortical tissue by Western blot analysis. Although nitrotyrosine is not a specific marker for the *in vivo* generation of peroxynitrite, it has widely been used as a marker of oxidative and nitrosative stress [18]. We detected a major band of nitrated protein of unknown identity at around 55 kD position as it was previously demonstrated in the kidney tissue using the same antibody [19]. Considering this 55 kD protein, nitrotyrosine levels (expressed as a ratio of nitrotyrosine/actin in densitometric units) were found similar between WKY (1.0 ± 0.26) and SHR (1.32 ± 0.44) rats at 2 weeks of age (Figure 4). However, nitrotyrosine level was found elevated by 1.6 fold in 3-week-old SHR compared to age-matched

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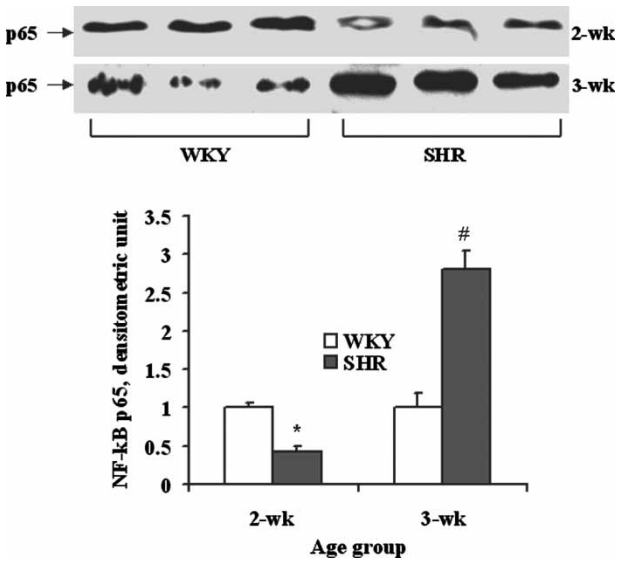

Figure 3. NF-κB activation as measured by intra-nuclear p65 protein levels during early development in SHR and age-matched WKY rats. Representative Western blots (upper panel) for SHR and WKY rats at 2 and 3 weeks of age. Densitometric analysis (lower panel) of intra-nuclear p65 in WKY and SHR rats. Equal loading was confirmed by Ponceau S stain. Values obtained in SHR groups are expressed relative to those of WKY groups, which are arbitrarily assigned a value of 1.0. Bars are representing means ± SD of at least three independent experiments ($n = 4$ in each group); $\star p = 0.003$ and $\#p = 0.001$ vs. age-matched WKY group.

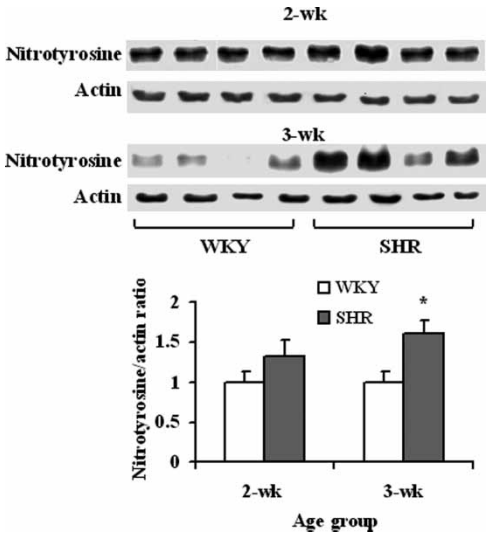

Figure 4. Renal cortical nitrotyrosine levels in SHR and age-matched WKY rats. Representative Western blots (upper panel) for SHR and WKY rats at 2 and 3 weeks of age. Actin was used as control for protein loading. Densitometric analysis of nitrotyrosine/actin ratio (lower panel) in WKY and SHR rats. Values obtained in SHR groups are expressed relative to those of WKY groups, which are arbitrarily assigned a value of 1.0. Bars are representing means ± SD of at least three independent experiments; $n = 5$ in each group; $\star p = 0.019$ vs. age-matched WKY group.

WKY rats (WKY: 1.0 ± 0.26 vs. SHR: 1.62 ± 0.29, $p = 0.019$) (Figure 4).

Oxidative stress-induced DNA damage. The DNA base guanine-containing nucleoside 2′-deoxyguanosine can be modified to 8-hydroxy-2′-deoxyguanosine (8-OHdG) by excessive oxidative stress [20]. We detected renal cortical cells containing the modified base 8-OHdG in the nucleus by immunohistochemistry using monoclonal antibody against 8-OHdG. The staining pattern was heterogeneous, involving tubular cells mainly in the inner cortex and medulla. There were different grades of positive staining, some nuclei were intensely stained and some others were faintly stained or stained with intermediate intensity (Figure 5). However, the median (range) number of cortical cells containing positively stained nuclei was not different between WKY (158(22–294)) and SHR (59(21–236)) rats at 2 weeks of age ($n = 5$). But the median number of cells containing positively stained nuclei were elevated in 3-week-old SHR compared to age-matched WKY rats (WKY: 27(25–119) vs. SHR: 191(64–492), $p = 0.049$, $n = 5$ in each group).

NADPH oxidase. NADPH oxidase is the major source of pro-oxidant superoxide in the vascular tissue [21]. This enzyme system has been found upregulated in different animal models of HTN associated with oxidative stress [9,19]. Recently, it has been shown that high blood pressure, *per se*, can promote the expression of NADPH oxidase subunits in the arterial wall [22]. To study the participation of this enzyme system in the pathogenesis of renal oxidative stress and associated inflammatory events in prehypertensive SHR, we investigated the expression of p47phox, a

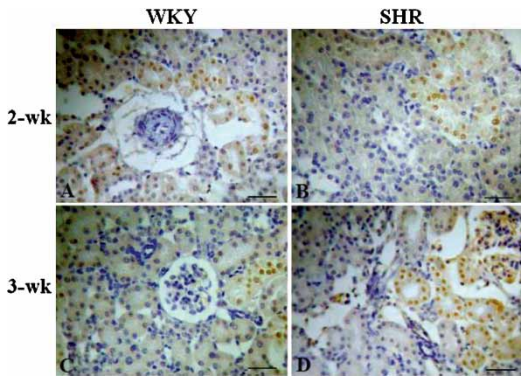

Figure 5. Immunohistochemical detection of 8-hydroxy-2′-deoxyguanosine (8-OHdG) in the kidney of WKY and SHR at 2 (A, B) and 3 weeks (C, D) of age. Positive staining was observed as brown-stained nuclei. Number of 8-OHdG positive cells was found elevated in 3-week-old SHR, but not in 2-week-old SHR, compared with age-matched WKY rats. Sections were counterstained with hematoxylin (original magnification × 400). Scale bar = 50 μm.

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regulatory subunit of NADPH oxidase, and Nox4, a homologue of gp91phox subunit of NADPH oxidase system.

The expression of p47phox in the renal cortex was found significantly elevated in 2-week-old SHR compared to age-matched WKY rats (WKY: 1.0 ± 0.29 vs. SHR: 1.75 ± 0.27, $p = 0.01$, expressed as a ratio of p47phox/actin in densitometric unit) (Figure 6). The expression of p47phox was found further elevated by more than 6 folds in 3-week-old SHR compared to age-matched WKY rats (WKY: 1.0 ± 0.47 vs. SHR: 6.59 ± 1.0, $p < 0.001$) (Figure 6). However, the expression of Nox4 was not different between SHR and WKY rats at 2- or 3-week time points (data not shown).

Reduced glutathione (GSH). Renal cortical GSH concentration was assessed as a measure of non-enzymatic antioxidant. GSH concentration (in micromole/g frozen tissue) was found elevated in SHR at 2 weeks (WKY: 3.08 ± 0.59 vs. SHR: 4.14 ± 1.03, $p = 0.013$, $n = 10$ in each group) and at 3 weeks (WKY: 4.49 ± 0.55 vs. SHR: 5.0 ± 0.47, $p = 0.038$, $n = 10$ in each group) of age compared to age-matched WKY rats. The increased GSH levels at 2 and 3 weeks of age in SHR may be an adaptive response to protect the kidney from underlying pro-oxidant stress.

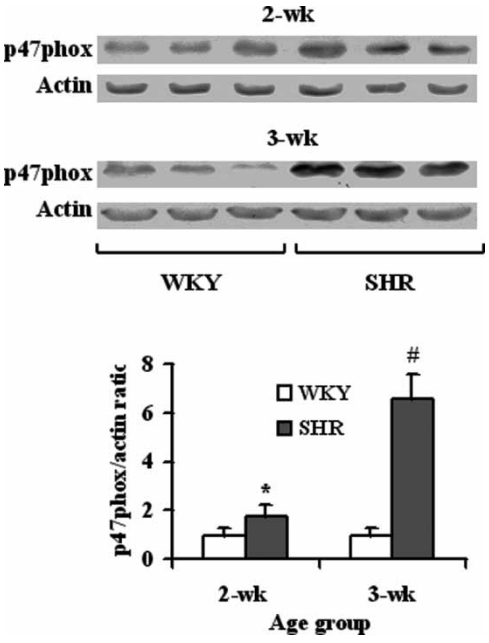

Figure 6. Expression of p47phox in the kidney cortex in SHR and age-matched WKY rats. Representative Western blots (upper panel) for SHR and WKY rats at 2 and 3 weeks of age. Actin was used as control for protein loading. Densitometric analysis of p47phox/actin ratio (lower panel) in WKY and SHR rats. Values obtained in SHR groups are expressed relative to those of WKY groups, which are arbitrarily assigned a value of 1.0. Bars are representing means ± SD of at least three independent experiments; $n = 5$ in each group; $\star p = 0.01$, and $\#p < 0.001$ vs. age-matched WKY group.

Antioxidant treatment

Antioxidant treatment with NAC and Tempol in 2-week-old SHR for 1 week did not alter systolic BP (control: 106 ± 9 vs. treated group: 103 ± 11 mm Hg, $n = 5$ in each group). However, renal cortical oxidative stress, as assessed by nitrotyrosine levels, was significantly reduced by antioxidant treatment (control: 1 ± 0.32 vs. treated group: 0.49 ± 0.21, nitrotyrosine/actin ratio, $p = 0.022$, $n = 5$ in each group, Figure 7). We evaluated renal cortical inflammatory process in the same group of rats to answer whether renal inflammation could be a consequence of renal oxidative stress in prehypertensive SHR. We found that the renal cortical macrophage infiltration in the tubulointerstitial area was significantly reduced in the treated group compared with control group (control: 22.45 ± 3.10/HPF vs. treated group: 18.17 ± 2.53/HPF, $p = 0.044$, $n = 5$ in each group, Figure 8). In addition, NF-κBp65 level in the renal cortical homogenate was also found significantly reduced in treated group compared with control group (control: 1 ± 0.31 vs. treated group: 0.63 ± 0.14, NF-κBp65/actin ratio, $p = 0.039$, $n = 5$ in each group, Figure 7). However, intra-nuclear fraction of p65 failed to show a significant decrement in the treated group (control: 1 ± 0.14 vs. treated group: 0.80 ± 0.39 densitometric unit).

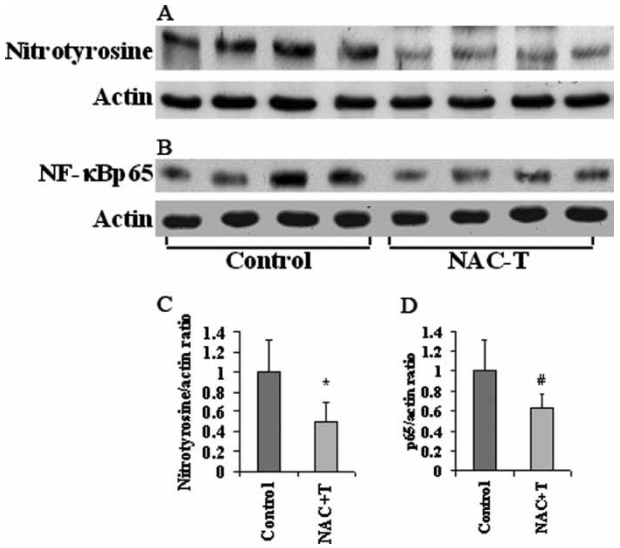

Figure 7. Renal cortical nitrotyrosine and NF-κB p65 in control and antioxidant treated (NAC + T) SHR rats. Representative Western blots for nitrotyrosine (A) and NF-κBp65 (B). Actin was used as control for protein loading. Densitometric analysis of nitrotyrosine/actin ratio (C) and p65/actin ratio (D). Value obtained in treated group is expressed relative to that of control group, which is arbitrarily assigned a value of 1.0. Bars are representing means ± SD of at least 3 independent experiments; $\star p = 0.022$ and $\#p = 0.039$ vs. control group; $n = 5$ in each group; NAC + T, *N*-acetyl-L-cysteine and Tempol.

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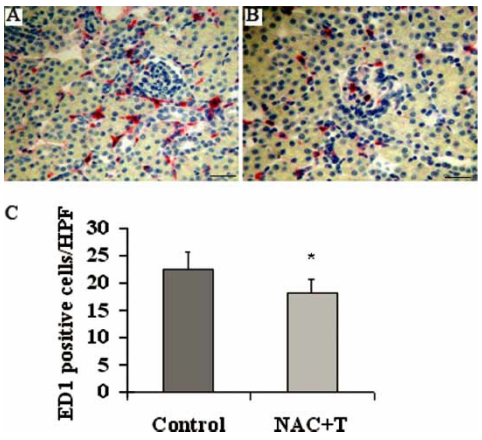

Figure 8. Immunohistochemical detection of macrophage (ED1-positive cell) infiltration in the kidney of control (A) and antioxidant treated (B) rat. Tubulointerstitial macrophage infiltration was evaluated by counting ED1-positive cells in 20 cortical high power fields and expressed as positive cells per high power field (C). Four sections from each animal were evaluated. Bars are representing means ± SD; $\star p = 0.044$ vs. control group, $n = 5$ in each group. NAC + T, *N*-acetyl-L-cysteine and Tempol; HPF, high power field. Sections were counterstained with hematoxylin (original magnification × 400). Scale bar = 50 μm.

Discussion

It is well accepted that the systemic HTN, renal inflammation and oxidative stress are tightly associated events in different models of HTN [4,8,23]. The association of these three events is not just a simple coexistence, rather they are closely interdependent in a manner that by modulating any of them the status of other two could be modulated, at least partly [2,3,6,7]. This fact has been highlighted in several recent reviews and the importance of identification of the primary event among HTN, renal inflammation and oxidative stress has been emphasized [8,23]. Since the events are interdependent, identification and targeting of the primary culprit may be of great clinical importance. Although, a great deal of work has already been done on this issue, the novelty of the present study is the simultaneous investigation of renal inflammation and oxidative stress at two different time points in very young prehypertensive SHR. Findings of our baseline study and antioxidant intervention suggest that the oxidative stress appears as a primary abnormality in the kidney that leads to renal inflammation before development of hypertension in SHR.

In the present work, we decided to study very young prehypertensive SHR of 2 and 3 weeks of age and age-matched WKY rats, because previous studies had confirmed that the BP does not differ between WKY and SHR rats at 2 and 3 weeks of age [5,24]. Although, the accuracy of the tail-cuff plethysmography has been questioned, the BP data obtained in the present study are similar to those obtained by Rodriguez-Iturbe et al. [5] and Dickhout and Lee [24]. The SHR rats studied were normotensive and the systolic BP of the SHR was similar to that of WKY rats both at 2 and 3 weeks of age. Therefore, we consider that the BP did not modify renal inflammation or oxidative stress in the animals we studied. According to the objective of the study, we did all experiments and comparisons in an age-matched fashion to explore the differences in renal inflammation and oxidative stress between SHR and age-matched WKY rats. The inflammatory/oxidative stress parameters we investigated are likely to be different between 2 and 3 week of age within a rat strain, however, we did not study this age effect in the present work.

The markers of renal inflammation and oxidative stress showed clear elevation in SHR at 3 weeks of age compared with age-matched WKY rats. However, at 2 weeks, there was no evidence of elevated renal inflammation in the SHR, as the macrophage infiltration was similar and intra-nuclear NF-κBp65 level was even lower in 2-week-old SHR compared to age-matched WKY rats. On the other hand, among the five oxidative stress markers studied, the expression of NADPH oxidase subunit p47phox showed significant elevation in the kidney of 2-week-old SHR compared to age-matched WKY rats.

We consider that the findings obtained at the 2-week time point need careful interpretation. Our data clearly indicate that the inflammatory process does not start in the kidney of 2-week-old SHR. The question is whether oxidative stress starts at the 2-week time point. Since oxidative stress is typically defined as a disturbance in the pro-oxidant/antioxidant balance in favor of the former, leading to potential damage, [25] our data do not show a direct evidence of oxidative stress at the 2-week time point. However, present data indicate that the upregulation of p47phox subunit of NADPH oxidase starts at (or before) the age of 2 weeks in the kidney, which shows marked elevation at the age of 3 weeks with associated oxidative damage. The elevation of the antioxidant GSH levels at the 2- and 3-week time points compared with age-matched WKY rats also provides functional evidence of enhanced pro-oxidant production in SHR.

To further elucidate whether inflammation or oxidative stress is the primary abnormality in the kidney in prehypertensive SHR, we treated 2-week-old SHR rats with antioxidants, NAC and Tempol. Although antioxidants are known to reduce BP in SHR, [2,7,19,26] we did not observe an alteration of BP with NAC and Tempol therapy for 1 week. This finding is not unlikely as we treated prehypertensive rather than hypertensive rats. Welch et al. [26], observed BP reduction in hypertensive SHR, but not in normotensive WKY rats, with oral Tempol therapy for 2-weeks. However, in the present study, antioxidant therapy reduced renal oxidative stress that was associated with a significant reduction of tubulointerstitial macrophage infiltration in the renal cortex.

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Total cortical NF-κBp65 level was also significantly reduced with antioxidant therapy, but the reduction of intra-nuclear fraction of p65 failed to reach the level of significance. Although we are unable to provide a definitive explanation of this last finding, the regulation of inflammatory gene expression by the NF- system is a complex issue and it does not solely depend on the p65 level or its transmigration into the nucleus. However, results of the antioxidant therapy suggest that oxidative stress, but not inflammation, is the primary abnormality in the kidney in prehypertensive SHR.

The NADPH oxidase enzyme complex is composed of membrane-bound subunits (gp91phox and p22phox) and cytosolic subunits (p47phox, p67phox and p40phox). Upon activation, some subunits, including p47phox, are phosphorylated and translocated to the membrane and participate in superoxide generation [27]. Chabrashvili et al. [9], previously showed that the p47phox expression is elevated in the kidney of 4-week-old SHR. They suspected from this finding that the oxidative stress in the kidney precedes the development of HTN in SHR [28]. However, several studies including our previous study have shown that the BP is already elevated in the SHR at 4-7 weeks of age compared with age-matched WKY rats [16,29]. Therefore, it was uncertain whether the over-expression of p47phox in the kidney of 4-week-old SHR was an effect of elevated BP in the study by Chabrashvili et al. [9]. The findings reported in the present study confirmed that the p47phox over-expression occurs in the kidney of SHR at 2 weeks of age, long before the development of HTN in this rat strain. In contrast to the findings of Chabrashvili et al. [9] and the present study, Zhan et al. [19] did not observe an overexpression of p47phox, instead they found overexpression of gp91phox and p22phox subunits in SHR. Although this discrepancy is difficult to explain, Zhan et al. [19] studied 24-week-old SHR that were hypertensive for a long duration.

Regarding the renal inflammatory process, similar to the findings of the present study, it has recently been shown that the renal interstitial inflammation and pro-inflammatory transcription factor NF-κB are already elevated in the prehypertensive SHR of 3-weeks of age [5]. However, this last study did not investigate the relation of oxidative stress with inflammation in the kidney of prehypertensive SHR. In the present study, to the best of our knowledge, for the first time we investigated the relationship between renal inflammation and oxidative stress in prehypertensive SHR, and we have identified primary abnormality among HTN, renal inflammation and oxidative stress in young SHR.

In conclusion, the present study apparently separates two closely interdependent and associated events, renal inflammation and oxidative stress, in SHR; and indicates that the oxidative stress appears before inflammation as a primary abnormality in the kidney in prehypertensive SHR. The identification of primary abnormality may have major implications in the prevention of HTN, because the renal oxidative stress and associated inflammation that appears before the development of HTN in SHR may be involved in the subsequent development of HTN and nephropathy.

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