Postnatal renin-angiotensin system inhibition prevents renal damage from prenatal inflammation in rats

Guo Wei, Ji Yan, Han Qi, Yang Yao, Wang Fangjie, Yang Yongjian, Deng Youcai, Sun Xiongshan, Li Xiaohui

Abstract

OBJECTIVE: To assess the protective role of benazepril, an angiotensin-converting enzyme inhibitor, in renal damage caused by prenatal inflammation.

METHODS: Saline or lipopolysaccharide were administered intraperitoneally to pregnant Sprague-Dawley rats on gestation days 8, 10, and 12. After birth, offspring received either tap water or benazepril in water between 7 and 68 weeks. Blood pressure, blood urea nitrogen, creatinine, and 24-h urine volume were measured as indices of renal function. Hematoxylin, eosin, periodic acid-Schiff, and Sirius Red staining were used to evaluate renal damage.

RESULTS: Postnatal benazepril treatment ameliorated hypertension and restored normal 24-h urine volume and blood urea nitrogen and serum creatinine levels. Benazepril treatment also reduced glycoprotein accumulation and fibrosis in the glomerulus and in tubular epithelial cells and inhibited nuclear factor-kappa B activation.

CONCLUSION: Together with our previous findings that postnatal inhibition of nuclear factor-kappa B activation blocks intra-renal renin-angiotensin system activation, our current data demonstrate that intra-renal activation of the renin-angiotensin system interacts with nuclear factor-kappa B activation to cause renal damage in adulthood following prenatal inflammation.

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Keywords: Renin-angiotensin system; NF-kappa B; Benazepril; Prenatal inflammatory exposure

INTRODUCTION

Hypertension is a major risk factor for cardiovascular and renal diseases and can lead to severe morbidity and mortality. Despite the availability of prevention strategies, the incidence of cardiovascular diseases is still increasing; preventing and treating these conditions re-
requires an understanding of their mechanisms. Clinical research has focused on factors such as obesity, diabetes, caffeine intake, infection, and inflammation in the development of chronic diseases. Epidemiological studies have shown that prenatal inflammatory challenges are associated with increased risk of cardiovascular diseases. We previously established an animal model of prenatal exposure to inflammation by lipopolysaccharide (LPS) administration at the second trimester, which leads to hypertension later in life. However, the underlying mechanisms are still largely unknown.

We previously reported that prenatal inflammation resulted in renal dysfunction (lower creatinine clearance rates, higher urinary protein levels, and renal fibrosis) in adult rats, and that intra-renal abnormal activation of the renin-angiotensin system (RAS) occurs in rats exposed prenatally to LPS. However, the role of intra-renal dysfunctional RAS activity in renal damage induced by prenatal inflammation is unclear. Abnormal RAS activation can lead to the development of cardiovascular conditions such as hypertension and heart failure and to chronic kidney disease. In addition to circulating RAS, RAS in local tissues such as heart, blood vessels, nerves, and kidneys can also progress cardiovascular diseases, providing a target for new therapies. Intra-renal RAS activation has been shown to induce renal damage and microvascular remodeling, and blockage of intra-renal RAS activity by an inhibitor of angiotensin-converting enzyme (ACE) can substantially inhibit hypertension. We previously showed that the kidneys of rats exposed prenatally to LPS expressed higher levels of renin and angiotensin (Ang) II postnatally through 25 weeks of age but without notable changes in the levels of circulating Ang II. We then found that blocking nuclear factor-kappa B (NF-kB) activation prenatally or postnatally using the specific 1kxIkB degradation inhibitor pyrrolidine dithiocarbamate restored normal ACE and Ang II protein levels, conferring renal protection. However, whether crosstalk occurs between abnormally expressed RAS and NF-kB activation in developmental kidney damage is unknown.

In the present study, we used the ACE inhibitor benazepril (BZ) to determine the role of intra-renal RAS activation in the progression of renal damage in rats exposed prenatally to LPS and its interaction with intra-renal NF-kB activation.

MATERIALS AND METHODS

Animals
Nulliparous pregnant time-mated Sprague-Dawley rats were obtained from the Experimental Animal Center of the Third Military Medical University (Chongqing, China). Animals were raised as previously described. Briefly, the rats were housed under a 12-h light/dark cycle, 22-25 °C, periodic air changes, and free access to water and food. The present study followed the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996; http://www.nap.edu/readingroom/books/labrats/index.html). All experiments performed in this study were approved by the local animal ethics committee at Army Medical University. Rats used in this study were anesthetized by pentobarbital [10 mg/kg body weight intraperitoneal injection (ip)] and then sacrificed by decapitation. The pregnant rats received saline (control) or 0.79 mg/kg LPS (Sigma-Aldrich, St. Louis, MO, USA) intraperitoneally on gestational days 8, 10, and 12, as described previously. Since both blood pressure and renal damage did not differ between male and female offspring, we selected an equal number of male and female offspring rats for each experiment in this study. Offspring were treated with BZ (120 mg/L; Yuancheng Technology Co., Ltd., Wuhan, China) through drinking water from postnatal weeks 7 to 68 by random selection. Offspring without additional treatment were used as vehicle controls. At 68 weeks of age, offspring were anesthetized with chloral hydrate (0.35 g/kg, 7% in saline) and sacrificed by decapitation. Kidneys were collected for analysis.

Immunoblotting
Kidney samples were lysed with T-PER™ tissue protein extraction reagent (Pierce, Rockford, IL, USA) with protease inhibitor cocktail (Sigma-Aldrich) and immunoblotting was performed as previously described. Antibodies against ACE (1:1000), Ang II (1:1000), NF-kB p65 (1:1000), phosphor (p)-p65 NF-kB (1:1000), and GAPDH (1:5000) were purchased from Cell Signaling Technology (Danvers, MA, USA).

Quantitative real-time polymerase chain reaction (qRT-PCR)
Total RNA was isolated by Trizol and then reverse-transcribed using a cDNA synthesis kit (Deutsche Biotech Innovativ AG, Berlin, Germany) and RT-PCR was performed as described previously. All primers used were described in our previous studies.

Analysis of urine volume
Each rat was kept in an individual metabolic cage to collect 24-h urine with free access to water and food for seven consecutive days prior to sacrifice. The mean 24-h urine volume was calculated from 7-d data.

Analysis of systolic blood pressure
Systolic blood pressure was analyzed using the noninvasive tail-cuff method with computer-assisted BP-2010 Series tail measurement equipment (Softron Beijing Biotechnology Co., Ltd., Beijing, China) as previously described.
**Analysis of blood urea nitrogen and serum creatinine**

Blood was taken from the inferior vena cava of rats. Blood urea nitrogen and serum creatinine were analyzed by an automatic biochemical analyzer at Xinqiao Hospital.

Histological evaluation of renal gross morphology by hematoxylin and eosin and periodic acid-Schiff staining Kidney samples from 68-week-old offspring were fixed with 4% paraformaldehyde, embedded in paraffin, and cut into 4-μm-thick sections. Hematoxylin and eosin staining was performed using our standard in-house protocol and periodic acid-Schiff staining was performed using a kit (Sigma-Aldrich) according to the manufacturer’s instructions.  

**Sirius red staining**

Slices were stained with Sirius Red saturation picric acid for 30 min following de-waxing and rehydration as described previously. Slides were then washed twice with water and nuclei were stained with hematoxylin. A light microscope (Olympus BH-2, Tokyo, Japan) and a polarized microscope (Olympus BX-51, Tokyo, Japan) were used for viewing the slides. Under light microscopy, collagen fibers appear in red. Under polarized microscopy, collagen type I fibers appear yellow and red with strong double refraction, while collagen type III fibers appear green with light double refraction.

**Statistical analysis**

For multiple comparisons, two-way analysis of variance (post hoc for least significant difference or Dunnett’s T3 test for inter-group comparison) was performed in SPSS 13.0 software (IBM SPSS, Armonk, NY, USA). Data are shown as mean ± standard deviation. \( P < 0.05 \) was considered statistically significant.

**RESULTS**

**BZ represses excessive RAS activation following prenatal LPS exposure**

As our laboratory previously reported increased expression of the intra-renal RAS components renin and Ang II between 7 and 25 weeks in rats prenatally exposed to LPS, we first assessed the effect of BZ on RAS components by determining ACE and Ang II protein expression by immunoblotting and mRNA expression of ACE, angiotensin (Ang), Renin, and angiotensin II type 1 receptor (AT1R) by qRT-PCR in the kidney tissues of 68-week-old rats after BZ treatment. As expected, prenatal inflammation induced significantly higher expression of intra-renal ACE and Ang II (Figure 1A) that were mainly distributed in the glomerulus, and BZ treatment reversed these changes. mRNA levels of renal ACE and Ang were also up-regulated by prenatal LPS stimulation and BZ treatment reduced over-expression (Figure 1B).

**BZ ameliorates prenatal LPS exposure-induced hypertension and restores normal urine volume, blood urea nitrogen, and serum creatinine in adult rats**

Consistent with our previous findings that prenatal LPS stimulation led to increased blood pressure, we confirmed that systolic blood pressure of rats that were exposed to LPS prenatally reached levels considered the

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![Figure 1](image-url)  

Figure 1: Postnatal benazepril treatment effectively blocks renal renin-angiotensin system expression in rats exposed prenatally to lipopolysaccharide. A1, A2: renal ACE and Ang II protein levels in 68-week-old rats were determined by western blotting (n = 3). 1: Con + Ve; 2: LPS + Ve; 3: LPS + BZ; 4: Con + BZ. \( p < 0.001 \), compared with the Con + Ve group; \( p < 0.001 \), compared with the LPS + Ve group. B: mRNA levels of ACE, Ang, renin, and AT1R in 68-week-old rats were determined by real-time quantitative polymerase chain reaction (n = 6). Two-way analysis of variance (post hoc for least significant difference test) was used for inter-group comparisons. Data are shown as mean ± standard deviation. BZ: benazepril; LPS: lipopolysaccharide; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; ACE: angiotensin-converting enzyme; Ang: angiotensin; Con: control; Ve: vehicle; AT1R: angiotensin II type 1 receptor. \( p < 0.05 \), compared with the Con + Ve group; \( p < 0.05 \), compared with the LPS + Ve group.
standard of hypertension, and this increase was suppressed by postnatal BZ treatment (Figure 2A).

Decreased urine volume is a typical symptom of renal damage and is thus regarded as a direct indication of renal dysfunction.16 We found that the volume of 24-h urine was significantly lower in 68-week-old rats that were exposed to LPS prenatally, and this decline was suppressed by BZ treatment (Figure 2B).

Increased levels of blood urea nitrogen and serum creatinine are classical indices of chronic renal damage.17 Postnatal BZ application suppressed these increases in blood urea nitrogen (Figure 2C) and serum creatinine (Figure 2D) in 68-week-old rats that were exposed prenatally to LPS.

**Postnatal BZ treatment prevents alterations in renal gross morphology in rats exposed prenatally to LPS**

To evaluate renal structural damage in rats exposed prenatally to LPS after BZ treatment, we performed hematoxylin and eosin staining on kidneys at 68 weeks of age. The exposure resulted in significant inflammatory cell infiltration and swelling of the glomerulus and proximal tubules, and these pathological alterations were almost completely prevented by BZ treatment. Renal structure did not differ between control rats (no LPS exposure) and control rats that received BZ (Figure 3A).

To assess glycoprotein accumulation in the glomerulus and in tubular epithelial cells, we performed periodic acid-Schiff staining. Consistent with the findings of our previous study, increased glycoprotein accumulation was noted in the glomerulus and in tubular epithelial cells of rats exposed prenatally to LPS. The accumulation was indicated by increased mesangial matrix in the glomeruli, and more sclerotic glomeruli were observed.18 Postnatal BZ treatment suppressed these pathological alterations (Figure 3B).

**Inhibition of RAS activation protects rats exposed prenatally to LPS from renal fibrosis**

Over-expression of RAS components has been linked to the progress of renal fibrosis,24,25 so we next determined whether postnatal BZ treatment reverses renal fibrosis in rats exposed prenatally to LPS. We observed larger areas that were Sirius Red-positive in both the glomeruli and interstitium of rats exposed prenatally to LPS, indicating increased deposition of collagen type I and III at 68 weeks of age; BZ treatment reversed or inhibited fibrosis (Figure 3C). Since Sirius Red staining mainly identifies collagen type I and type III, we also analyzed mRNA levels of collagen type I alpha 1 chain (Colla1) and collagen type III alpha 1 chain (Col3a1).26 As expected, rats exposed prenatally to LPS exhibited increased expression of renal Colla1 and Col3a1, which was abolished by postnatal BZ treatment (Figure 3D).

**BZ represses NF-κB-mediated inflammation in rats exposed prenatally to LPS**

Previous studies have reported that activation of NF-κB by Ang II leads to the expression of inflammatory cytokines such as interleukin (IL) 6 and monocyte chemoattractant protein 1 (MCP-1),27,28 which are involved in renal dysfunction.29 We determined NF-κB activity by immunoblotting of p-p65 and p65 and quantification of mRNA levels of its downstream

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**Figure 2 Postnatal BZ treatment ameliorates renal dysfunction in rats exposed prenatally to LPS**

A: mean SBP was measured at 68 weeks of age (n = 8); *P < 0.01, compared with the Con + Ve group; "P < 0.01, compared with the LPS + Ve group. B: Twenty-four-hour urine was collected for seven consecutive days from each rat and mean 24-h urine volume was calculated (n = 16). *P < 0.01, compared with the Con + Ve group. "P < 0.05, compared with the LPS + Ve group. C: Blood urea nitrogen (n = 16) and serum creatinine (n = 16) were measured at 68 weeks of age. *P < 0.01, compared with the Con + Ve group; "P < 0.01, compared with the LPS + Ve group. Two-way analysis of variance (post hoc for least significant difference test) was used for inter-group comparisons (A, C, and D); Dunnett’s T3 test was used in B. Data are presented as mean ± standard deviation. BZ: benazepril; LPS: lipopolysaccharide; SBP: systolic blood pressure; Con: control; Ve: vehicle.
Figure 3 Postnatal BZ treatment ameliorates renal morphological alterations in rats exposed prenatally to LPS
A1-A8: HE staining in kidney of Con + Ve, LPS + Ve, LPS + BZ, Con + BZ in week 68; A1-A4: magnification 100×; A5-A8: magnification 400×. B1-B8: PAS staining in kidney of Con + Ve, LPS + Ve, LPS + BZ, Con + BZ in week 68; B1-B4: magnification 100×; B5-B8: magnification 400×. C1-C8: Sirius Red staining in kidney of Con + Ve, LPS + Ve, LPS + BZ, Con + BZ in week 68; C1-C4: light microscope result (400×); C5-C8: polarized microscope result (400×); D: mRNA levels of renal Col1α1 and Col3α1 in 68-week-old rats were measured by real-time quantitative polymerase chain reaction (n = 6). Two-way analysis of variance (post hoc for Dunnett’s T3 test in D) was used for inter-group comparisons. Data are presented as mean ± standard deviation. *P < 0.05, compared with the Con + Ve group; †P < 0.05, compared with the LPS + Ve group. BZ: benazepril; LPS: lipopolysaccharide; Con: control; Ve: vehicle; PAS: Periodic acid-Schiff; HE: Hematoxylin and eosin.
we and 25 weeks of age, a model of chronic kidney damage for studying the un-
mulation in newborns, and the present study found that these effects were sustained at
weeks of age. Prenatal exposure to LPS can therefore be used as a
exposure can result in abnormal renal development and
ER and NF-kB activation may function as a
case, RAS and NF-kB activation may function as a
caused the inflammation induced by LPS. Since inhibi-
opinion factors, as evidenced by lower levels of p-p
vation of NF-kB and its downstream pro-inflam-
chronic kidney damage. BZ treatment ameliorated renal dam-
expression contributes to the development of chronic kidney damage. BZ treatment ameliorated renal dam-
chronic kidney damage. BZ treatment ameliorated renal dam-
reducing mesangial matrix deposition and glycogen accumulation in both the
glomerulus and interstitium. Chronic kidney damage is typically accompanied by re-
was perhaps caused by NF-kB activation, because this over-expression was
suppressed by an IκB degradation inhibitor. Previous research demonstrated that NF-kB-mediated up-regulation of inflammatory factors could also be in-
duced by local tissue levels of Ang II regarded as a
pro-inflammatory molecule that regulates various cellular
that in turn aggravates RAS activity and ultimately leads to
renal fibrosis and dysfunction in adulthood. Inhibition of abnormal RAS activity in development and early life
likely prevent chronic kidney damage.

**DISCUSSION**

The prenatal environment has been shown to affect
health in adulthood, and more research has focused on
preventing chronic renal diseases at early life stages. Pre-
vious studies have reported that adverse prenatal stimula-
tion such as nutrient restriction and corticosterone ex-
posure can result in abnormal renal development and
hypertension. Our laboratory previously reported that prenatal LPS stimulation resulted in reduced glo-
merular filtration rate, hypertension, reduced urine vol-
ume, structural damage, and matrix glycoprotein accu-
mulation in newborns, and the present study found that
these effects were sustained at 68 weeks of age.3,4
Prenatal exposure to LPS can therefore be used as a
model of chronic kidney damage for studying the un-
underlying mechanisms and prevention strategies.

Our previous study reported abnormally activated RAS 
between 7 and 25 weeks of age, and the current study 
found that RAS was still activated at 68 weeks. We 
used the ACE inhibitor BZ to demonstrate that prena-

Figure 4 Postnatal BZ treatment blocks NF-κB-mediated renal inflammation in rats exposed prenatally to LPS
A1, A2: expression of p-p65ser536 and total p65 was assessed by immunoblotting in kidneys of 68-week-old rats. Representative 
blot and relative densitometry normalized by GAPDH (A1) are shown (n = 5). 1: Con + Ve; 2: LPS + Ve; 3: LPS + BZ; 4: Con + BZ. 'P <
0.001, compared with the Con + Ve group; 'P < 0.001 and 'P < 0.05, compared with the LPS + Ve group. B: renal mRNA expression of
TNF-α, IL-6, IL-1β, and MCP-1 was determined by real-time quantitative PCR in rats at 68 weeks of age (n = 6). 'P < 0.05, com-
pared with the Con + Ve group; 'P < 0.01 and 'P < 0.05, compared with the LPS + Ve group. Two-way analysis of variance (post hoc 
for least significant difference test) was used for inter-group comparisons. Data are presented as mean ± standard deviation. BZ: 
benazepril; LPS: lipopolysaccharide; Con: control; VE: vehicle; NF-kB: nuclear factor-kappa B; GAPDH: glyceraldehyde-3-phosphate 
derhydrogenase; TNF-α: tumor necrosis factor alpha; IL-6: interleukin 6; MCP-1: monocyte chemoattractant protein 1.

**Figure 4** Postnatal BZ treatment blocks NF-κB-mediated renal inflammation in rats exposed prenatally to LPS

A1, A2: expression of p-p65ser536 and total p65 was assessed by immunoblotting in kidneys of 68-week-old rats. Representative 
blot and relative densitometry normalized by GAPDH (A1) are shown (n = 5). 1: Con + Ve; 2: LPS + Ve; 3: LPS + BZ; 4: Con + BZ. 'P <
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for least significant difference test) was used for inter-group comparisons. Data are presented as mean ± standard deviation. BZ: 
benazepril; LPS: lipopolysaccharide; Con: control; VE: vehicle; NF-kB: nuclear factor-kappa B; GAPDH: glyceraldehyde-3-phosphate 
derhydrogenase; TNF-α: tumor necrosis factor alpha; IL-6: interleukin 6; MCP-1: monocyte chemoattractant protein 1.
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