Oxidative Stress Mediates the Stimulation of Sympathetic Nerve Activity in the Phenol Renal Injury Model of Hypertension

Shaohua Ye, Huiquin Zhong, Vito M. Campese

Abstract—Renal injury caused by the injection of phenol in the lower pole of one kidney increases blood pressure (BP), norepinephrine secretion from the posterior hypothalamic nuclei (PH), and renal sympathetic nerve activity in the rat. Renal denervation prevents these effects of phenol. We have also demonstrated that noradrenergic traffic in the brain is modulated by NO and interleukin-1β. In this study, we tested the hypothesis that the increase in sympathetic nervous system (SNS) activity in the phenol renal injury model is because of activation of reactive oxygen species. To this end, we first examined the abundance of several components of reduced nicotinamide adenine dinucleotide phosphate oxidase (identified as the major source of reactive oxygen species), including gp91phox/Nox2, p22phox, p47phox, and Nox3 using real-time PCR. Second, we evaluated the effects of 2 superoxide dismutase mimetics, tempol (4-hydroxy-2,2,6,6-tetramethyl piperidinoxyl), and superoxide dismutase-polyethylene glycol on central and peripheral SNS activation caused by intrarenal phenol injection. Intrarenal injection of phenol raised BP, NE secretion from the PH, renal sympathetic nerve activity, and the abundance of reduced nicotinamide adenine dinucleotide phosphate and reduced the abundance of interleukin-1β and neural-NO synthase mRNA in the PH, paraventricular nuclei, and locus coeruleus compared with control rats. When tempol or superoxide dismutase-polyethylene glycol were infused in the lateral ventricle before phenol, the effects of phenol on BP and SNS activity were abolished. The studies suggest that central activation of the phenol renal-injury model is mediated by increased reactive oxygen species in brain nuclei involved in the noradrenergic control of BP. (Hypertension. 2006;48:309-315.)

Key Words: hypertension, renal sympathetic nervous system, nitric oxide, interleukins, oxidative stress

The kidney is a sensory organ richly innervated with both mechanosensitive and chemosensitive afferent nerve fibers, and renal afferent nerves project directly to a number of areas in the central nervous system contributing to arterial pressure regulation.7,8 Renal injury caused by the injection of phenol in the lower pole of one kidney caused an immediate and persistent elevation of blood pressure (BP), norepinephrine (NE) secretion from the posterior hypothalamic nuclei (PH), and renal sympathetic nerve activity (RSNA) in the rat.9,10 Renal denervation prevented the effects of renal injury on BP, NE secretion from the PH, and RSNA.

Considerable attention has been given to the effects of short-lived reactive oxygen species (ROS) and reactive nitrogen species on BP and cardiovascular toxicity. ROS or oxygen free radicals are O₂ molecules with an unpaired electron and include superoxide anion (O₂⁻), hydrogen peroxide, and hydroxyl ion. These molecules are chemically unstable and highly reactive, and NADPH oxidase, xanthine oxidase, and NO synthase (NOS) enzymes regulate their concentration. NADPH oxidase is a multimeric enzyme and is responsible for the reduction of oxygen, electron transport, and superoxide production at the cell surface.11 The phagocyte NADPH oxidase is composed of 2 essential membrane-bound components, gp91phox/Nox2 and p22phox, and 4 cytosolic components, p47phox, p67phox, p40phox, and Rac1.12 On stimulation, the 4 cytosolic components translocate to the membrane and increase the activity of NADPH oxidase. In nonphagocytic cells, 4 homologues of gp91phox/Nox2 have been identified, called Nox1 and Nox3 to 5.13

ROS production is increased in several experimental models of hypertension14–16 and in human hypertension.17 A causative role of ROS is supported by evidence that scavengers of ROS, such as dimercaptosuccinic acid, l-azadinos, cledalenine, and tempol (as superoxide dismutase mimetic), and vitamin C and E ameliorate or abrogate hypertension in animal models,18,19 Conversely, depletion of glutathione, an endogenous scavenger of ROS, by means of the glutathione synthase inhibitor, buthionine sulfoximine, caused a marked elevation of nitrotyrosine, the footprint of peroxynitrite, and marked elevation of BP in rats.20 The exact mechanisms through which oxidative stress may raise BP have not been fully elucidated, but reduced availability of NO is the prevailing hypothesis. Evidence sug-
gests that ROS may raise BP through activation of the sympa-
thetic nervous system (SNS). Because NO exerts a tonic inhi-
bition on central SNS activity, increased production of ROS may
activate the SNS through oxidation/inactivation of NO. NO
actively reacts with O₂ and other ROS to produce peroxynitrate,
a highly cytotoxic reactive nitrogen species. In this study, we
have tested the hypothesis that the increase in SNS activity in
the phenol renal injury model is because of activation of ROS. To
test this hypothesis, first we examined the abundance of several
components of NADPH oxidase, including gp91phox/Nox2,
p22phox, p47phox, and Nox3 in the PH, paraventricular nuclei
(PVN), and locus coerulescens (LC) using real-time PCR. Second,
we evaluated the effects of 2 superoxide dismutase mimetic,
tempol (4-hydroxy-2,2,6,6-tetramethyl piperidinoxyl), and su-
peroxide dismutase (SOD)-polyethylene glycol (SOD) on cen-
tral and peripheral SNS activation caused by intrarenal phenol
injection.

Methods

Animals and Surgical Methods

For these studies, we used male Sprague–Dawley rats weighing 200
to 250 g fed with normal rat chow (ICN Nutritional Biochemical)
and tap water. For measurements of arterial pressure and adminis-
tration of drugs, we anesthetized animals with sodium pentobarbital
(35 mg/kg IP followed by an infusion of 5 mg/kg per hour) and
implanted catheters (PE-10) in a femoral artery and vein.

The Phenol–Renal–Injury Model

After a dorsal incision, we exposed the left kidney and injected 50 μL of
10% phenol in the lower pole. Control rats received 50 μL of normal
saline in the lower pole of the kidney. Arterial pressure (BP) was
continuously recorded (Physiograph, Grass Instrument). As a marker
of central noradrenergic trafficking, we measured NE secretion from
the PH before the intrarenal injection of phenol and every 5 minutes
for 60 minutes thereafter. As a measure of peripheral SNS activity,
we continuously measured RSNA before the intrarenal injection of
phenol and for 60 minutes thereafter.

NE Secretion From the PH Using the
Microdialysis Technique

To measure NE secretion from the PH, we placed rats in a stereotoxic
apparatus, implanted a 2-mm long Teflon 22-gauge guide cannula (IV
Catheter Placement Unit; Critikon, Inc) using coordinates anterior-
posterior (A-P) at −4.2 mm, lateral at 0.4 mm, and vertical (V) at 8 mm
and secured the guide in place with dental cement. A 28-gauge stainless
steel stylus was lowered through the guide cannula to a depth 1.5 mm
alter BP, NE secretion from the brain, or RSNA.24. In a subset of 5 rats,
we infused PEG-SOD (in dose of 80 U/kg dissolved in 10
μL of body weight per minute) starting 15 minutes before the
intrarenal injection of phenol or saline and continuously for 60 minutes
thereafter. We evaluated the effects on BP, NE secretion from the PH,
and RSNA. Because we have shown previously that Tempol in the dose
used in this study by itself lowers BP and to test the specificity of tempol
effects, in separate groups of rats we evaluated the effects of a different
SOD mimetic, PEG-SOD (in dose of 80 U/kg dissolved in 10 μL of
aCSF, infused over 10 minutes) on BP, NE secretion from the PH, and
RSNA when given 15 minutes before the intrarenal injection of phenol.
We have shown previously that this dose of PEG-SOD by itself does not
alter BP, NE secretion from the brain, or RSNA.23 In a subset of 5 rats,
we infused PEG-SOD (in dose of 80 U/kg dissolved in 10 μL of aCSF)
30 minutes before the intrarenal injection of phenol and we evaluated
the effects on BP, NE secretion from the PH, and RSNA.

Determination of nNOS and Interleukin-1β mRNA
Abundance in the Brain

At the end of the experiments, rats were euthanized by decapita-
tion and brains immediately removed, frozen in dry ice, and stored at
−70°C until assay but for no longer than 3 weeks. Brains were cut
into consecutive 200-μm sections in a cryostat at −20°C and
bilateral micropunches 0.5 mm in diameter from several brain nuclei
obtained as described previously.24 In the experiments where micro-
dialysis was performed, we isolated the nuclei from the contra lateral
side to that of the implantation of the microdialysis probe.

The coordinates for the PH were: A-P, −3.5 to −4.1 mm; lateral,
0.3 mm; and V, 8 mm; for the LC were: A-P, from −1.4 to 2.0 mm;
lateral, ±0.3 mm; and V, 7.9 mm; and for the LC were: A-P, from
−9.8 to 10.2 mm; lateral, ±1.4 mm; and V, 7.2 mm.
The nuclei so isolated were used to measure IL-1β and neural-NOS (nNOS) mRNA gene expression. We selected those 5 nuclei because they are all involved in the noradrenergic control of BP.

Total RNA extraction and real-time reverse transcription (RT) were performed by methods described previously by us.24 PCR was performed on the RT product using specific oligonucleotide primers for either nNOS or interleukin (IL)-1β derived from cDNAs cloned from rat brain25 (Genbank, accession X59949) or rat liver.26 A master mix of PCR reagents was made for duplex reactions containing primers for the housekeeping gene β-actin (Genbank accession Joo691) and primers for either neuronal NOS (Genbank, accession X59949) or IL-1β (Accession M98820).

The RT-PCR products were quantified by the method of Higuchi and Dollinger.27 Fluorescence was measured in a fluorescence spectrofluorometer (F-2000, Hitachi Ltd). Excitation was at 280 nm, and emission was at 390 nm. Results were expressed as a ratio of the resultant optical densities for the specific gene to β-actin.

Random hexamers, DTT, Super Scrip Super RT with reaction buffer (×5; 20 mmol/L Tris-HCl, 10 mmol/L NaCl, 0.1 mmol/L EDTA, 1 mmol/L DTT, 0.01% NP-40, and 50% glycerol), TaqDNA polymerase with reaction buffer (×10; 50 mmol/L Tris-HCl, 10 mmol/L NaCl, 0.1 mmol/L EDTA, 5 mmol/L DTT, 50% glycerol, and 1.0% Triton X-100), 2′-deoxynucleoside 5′-triphosphate, and MgCl2, were purchased from GIBCO/BRL.

**Determination of Catalytic Components of NADPH Oxidase in Brain Nuclei**

Total RNA was prepared as described above. RT-PCR was performed with gene-specific primers as shown in the Table, using the same methods described for nNOS. We measured several components of the NADPH oxidase, including rat p22-phox, p47 phox, gp91phox/Nox2, and Nox3.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Forward (5′-3′)</th>
<th>Reverse (5′-3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>r p22phox</td>
<td>TGCTCTGATCTCTGACAG</td>
<td>AGGCAGCAAGACACGATAG</td>
</tr>
<tr>
<td>r p47phox</td>
<td>TCACCAGATCTACAGCTTC</td>
<td>TCCCTAGAGCGCTTGAAGT</td>
</tr>
<tr>
<td>r Nox3</td>
<td>GAGTGGCACCCCTTCACCCT</td>
<td>CTAGAAGACCCGTCTTTG</td>
</tr>
<tr>
<td>r gp91phox/Nox2</td>
<td>CCAGTGAAGATGTGTTCAGCT</td>
<td>GCCAGCCAAGATAGAT</td>
</tr>
</tbody>
</table>

r indicates rat.

**Location of Probe**

At the end of the experiments, although rats were still anesthetized, the dialysis probes were removed, and rats were euthanized by decapitation. Brain were immediately removed, frozen in dry ice, and stored at −70°C. Later, brains were sliced in 200-μm sections and stained with cresyl violet to localize the tip of the probe. Only rats with probes properly implanted in the PH were considered for further analysis. Approximately 15% to 20% of animals were eliminated because of improper positioning of the probes.

**NE Microassay**

We used a highly sensitive microradioenzymatic assay.28 A total of 10 μL of dialysate was added to 5 μL of reaction mixture containing 1 μL of 3.7 mol/L Tris base (with 0.37 mol/L EGTA and 1.8 mol/L MgCl2; pH 8.2), 0.06 μL of 36 mmol/L benzoylamine, 1.5 μL of S-[methyl-3H] adenosyl-L-methionine, and 2.4 μL of partially purified catechol-O-methyltransferase and incubated for 60 minutes at 37°C. The sensitivity of this method is 0.5 pg.

**Statistical Analyses**

Data were analyzed by 1-way ANOVA and by Fisher’s exact test when indicated. A 2-way ANOVA was used to examine interactions among the effects of phenol injection, tempol, and PEG-SOD. The computer program Prism (GraphPad Software) was used for the analyses. Results are expressed as mean±SEM.

**Results**

**Interaction of Tempol and PEG-SOD With the Phenol-Renal Injury–Induced Effects on BP NE Secretion From the PH and RSNA**

Intrarenal injection of phenol significantly (P<0.01) raised the mean BP, RSNA, and NE secretion from the PH (Figures 1–3). By contrast, the administration of saline in the kidney caused no change in BP or NE secretion from the PH.

When tempol (50 μg/μL per kilogram per minute dissolved in aCSF) was given intracerebroventriculatively 15 minutes before the phenol injection, the effects of phenol on BP and NE secretion from the PH and RSNA, after an initial surge, were completely abolished (Figures 1–3).

Because we showed previously that at the dose of 50 μg/μL per kilogram per minute, tempol by itself reduced BP,
NE secretion from the PH, and RSNA, this might suggest that the ability of tempol to block the effects of phenol-renal injury on BP, NE secretion, and RSNA may not necessarily depend on interference with renal injury-mediated ROS activation. Instead, renal injury and tempol could have independent and opposite effects on BP.

To partially deal with this possibility, we used a different SOD agonist: PEG-SOD. PEG-SOD given alone in a dose of 80 U/kg of body weight has no direct effects on BP, NE secretion from the PH, and RSNA. However, this dose of PEG-SOD, after an initial surge, significantly attenuated the effects of phenol-induced renal injury on BP, NE secretion from the PH, and RSNA (Figures 1–3).

Because injecting PEG-SOD 15 minutes before the injection of phenol might not give the drug the necessary time to fully activate enzyme activity, in a subset of rats, we repeated the same experiments, but we infused the same dose of PEG-SOD 30 minutes before the intrarenal injection of phenol. In this case, the initial surge in BP, NE secretion from the PH, and RSNA were completely abolished (Figures 1–3). There was a statistically significant interaction (P<0.001×2-way ANOVA) among PEG-SOD, renal injury, and BP, NE secretion from the PH, and RSNA.

**Effects of Tempol, PEG-SOD, and Renal Injury on nNOS and IL-1β Abundance in the PH, PVN, and LC**

The intrarenal injection of phenol significantly (P<0.01) reduced the abundance of IL-1β in the PH (from 75.0±2.3 to 37.0±1.6), PVN (from 76±2.4 to 45.2±1.8), and LC (from 56.1±2.3 to 42.5±1.8) compared with control rats (Figure 4B). Intrarenal phenol also significantly (P<0.01) reduced the abun-
Effects of Intrarenal Phenol on the Abundance of NADPH Subunits in Brain Nuclei

The intrarenal injection of phenol caused a significant increase in the abundance of several components of NADPH, including gp91phox/Nox2, p22phox, nox3, and p47phox (Figure 5).

Discussion

These studies have confirmed that an intrarenal injection of phenol raises BP, NE secretion from the PH, and RSNA and reduces the abundance of nNOS and IL-1β in several brain nuclei. Tempol and PEG-SOD, 2 SOD mimetics, abolished the effects of intrarenal phenol injection on BP, NE secretion from the PH, and RSNA and on nNOS and IL-1β. In addition, the intrarenal injection of phenol caused a significant increase in the abundance of several components of NADPH in a variety of brain nuclei involved in the noradrenergic control of BP. The data support the hypothesis that ROS may modulate central and peripheral SNS activation that follows intrarenal phenol injection.

Tempol is a membrane-permeable and metal-independent SOD mimetic that has been widely used for the removal of intracellular and extracellular O$_2^-$, and has proven antioxidant activity in various tissues. In coronary arteries, O$_2^-$ has been shown to inactivate NO, and O$_2^-$ is important in the decomposition of NO to peroxynitrite.

The effects of tempol on BP have been controversial. Large doses of tempol given intravenously have been shown to acutely lower BP in normotensive and hypertensive rats and result in reflex activation of the SNS. By contrast, Xu et al. observed that tempol (300 μmol/kg IV) lowered BP and RSNA in urethane-anesthetized DOCA–salt-hypertensive rats. In a previous study, we observed that tempol infused intracerebroventricularly reduced BP, NE secretion from the PH, and RSNA. By contrast, Shokoji et al. observed no effects of tempol on BP and RSNA when bolus doses of this agent were infused ICV in SHR and WKY rats. Differences in dosing, type of administration, and animal model could explain the difference in results among these studies.

To further substantiate a role for O$_2^-$ in renal injury–mediated activation of SNS, we evaluated the effects of a different SOD agonist, PEG-SOD. PEG-SOD in a dose of 80 U/kg of body weight infused intracerebroventricularly caused no direct ef-
Effects on BP and SNS activity. However, when administered 15 minutes before the intrarenal injection of phenol, PEG-SOD reduced the effects of renal injury on BP, NE secretion from the PH, and RSNA. When we infused PEG-SOD 30 minutes before the intrarenal injection of phenol, the effects of renal injury on BP, NE secretion from the PH, and RSNA were completely abolished.

NOS is present in a specific area of the brain involved in the noradrenergic control of BP, and it is an important component of transduction pathways that tonically inhibit sympathetic nerve activity.27 NO actively reacts with O2- and other ROS to produce peroxynitrite, a highly cytotoxic reactive nitrogen species. Increased production of ROS could enhance oxidation/inactivation of NO and result in activation of the SNS. The current studies support this possibility. Intrarenal phenol reduced the abundance of nNOS in brain nuclei involved in the noradrenergic control of BP resulting in increased SNS activity and BP. By contrast, intracerebroventricular infusion of tempol and PEG-SOD abrogated these effects of phenol. This lends further support to our hypothesis that oxygen radicals may modulate central and peripheral SNS activation caused by intrarenal phenol injection.

We did not measure directly ROS production in the brain but, as an indirect marker, we measured several components of the NADPH oxidase, including rat p22-phox, p47 phox, gp91phox/Nox2, and Nox3. The abundance of all of these components was significantly raised in rats injected with phenol compared with control animals. This lends further support to our hypothesis that activation of SNS activity in the phenol-renal injury model is mediated by increased local production of ROS.

We have shown previously that IL-1β plays a modulatory role on the SNS. Administration of IL-1β in the lateral ventricle of 5 of 6 nephrectomized and control rats caused a dose-dependent decrease in BP and NE secretion from the PH, and an increase in nNOS-mRNA abundance in several brain nuclei.39 By contrast, intracerebroventricular infusion of a specific antirat IL-1β antibody decreased NOS-mRNA expression in the PH, PVN, and LC and raised BP and NE secretion from the PH in these rats. In the present studies, phenol injection in the kidney reduced the abundance of IL-1β and nNOS mRNA in the PH, PVN, and LC. By contrast, tempol and PEG-SOD abolished the effects of phenol injection on IL-1β. This suggests that ROS may inhibit IL-1β and nNOS production at a transcriptional level and participate in the central activation of the SNS in the phenol-renal injury model.

We have hypothesized previously that locally produced angiotensin II (Ang II) in the brain may mediate central activation of the SNS in the phenol-renal injury model. Losartan, an Ang II type 1 (AT-1) receptor antagonist, completely reversed the effects of phenol-renal injury on BP and SNS activity.40 This hypothesis is supported by evidence that locally produced Ang II may activate SNS activity in the central nervous system via increased superoxide generation.41–43

In summary, these studies support the hypothesis that a renal injury caused by intrarenal injection of phenol activates the production of ROS in specific brain regions leading to activation of the SNS and hypertension. The data also support the hypothesis that downregulation of nNOS and IL-1β may mediate the central effects of ROS.

Perspectives
Hypertension associated with kidney disease is very prevalent and difficult to control with currently available antihypertensive agents. The phenol-renal injury model offers a unique opportunity to study the mechanisms of hypertension associated with renal injury. The current studies extend previous observations that renal injury may activate afferent pathways connecting with brain regions involved in the noradrenergic control of BP. We have shown previously that locally activated Ang II in the brain may mediate central sympathetic activation caused by renal injury through increased oxidative stress. The current studies have shown that tempol and PEG-SOD attenuate the effects of renal injury on central SNS activation and BP, lending further support to our hypothesis that increased oxidative stress in brain regions involved in the noradrenergic regulation of BP mediates the effects of renal injury on SNS activity and BP.

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Disclosures
None.

References


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In a *Hypertension* article by Ye et al (Ye S, Zhong H, Campese VM. Oxidative stress mediates the stimulation of sympathetic nerve activity in the phenol renal injury model of hypertension. *Hypertension*. 2006;48:309–315), an author was omitted. The correct author list is Shaohua Ye, Huiquin Zhong, Swati Yanamadala, and Vito M. Campese. The authors regret the error.