Oxidative Stress

Selective Nrf2 Gene Deletion in the Rostral Ventrolateral Medulla Evokes Hypertension and Sympathoexcitation in Mice

Lie Gao, Matthew C. Zimmerman, Shyam Biswal, Irving H. Zucker

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Abstract—Nuclear factor erythroid 2–related factor 2 (Nrf2) is a master transcriptional regulator of redox homeostasis that impacts antioxidant gene expression. Central oxidative stress and reduced antioxidant enzyme expression in the rostral ventrolateral medulla (RVLM) contributed to sympathoexcitation in chronic heart failure. In the current study, we hypothesized that deletion of Nrf2 in the RVLM would increase sympathetic drive and blood pressure. Experiments were performed in Nrf2-floxed mice treated with microinjection of lentiviral-Cre-GFP or lentiviral-GFP into the RVLM. Two weeks after viral administration, Nrf2 message, protein, oxidative stress, cardiovascular function, and sympathetic outflow were evaluated. We found that (1) Nrf2 mRNA and protein in the RVLM were significantly lower in Cre mice compared with control GFP mice. Nrf2-targeted antioxidant enzymes were downregulated, whereas reactive oxygen species were elevated. (2) Blood pressure measurements indicated that Cre mice displayed a significant increase in blood pressure (mean arterial pressure, 123.7±3.8 versus 100.2±2.2 mm Hg; P<0.05, n=6), elevated urinary norepinephrine (NE) concentration (456.4±16.9 versus 356.5±19.9 ng/mL; P<0.05, n=6), and decreased spontaneous baroreflex gain (up sequences, 1.66±0.17 versus 3.61±0.22 ms/mm Hg; P<0.05, n=6; down sequences, 1.89±0.12 versus 2.98±0.19 ms/mm Hg; P<0.05, n=6). (3) Cre mice displayed elevated baseline renal sympathetic nerve activity and impaired inducible baroreflex function. These data suggest that Nrf2 gene deletion in the RVLM elevates blood pressure, increases sympathetic outflow, and impairs baroreflex function potentially by impaired antioxidant enzyme expression. (Hypertension. 2017;69:1198-1206. DOI: 10.1161/HYPERTENSIONAHA.117.09123.)

Key Words: arterial baroreflex ■ blood pressure ■ brain stem ■ oxidative stress ■ sympathetic regulation

Maintaining redox homeostasis is essential for normal physiological function. Increased production of reactive oxygen species (ROS) or impaired antioxidant defense mechanisms result in accumulation of ROS and oxidative stress, well documented to contribute to the pathogenesis of several chronic cardiovascular diseases, including heart failure and hypertension.1,2 The central nervous system is highly vulnerable to oxidative stress where excessive ROS can evoke oxidative modification of neuronal constituents, such as nucleic acids, lipids, and proteins, leading to acute and chronic functional and structural abnormalities.3,4 Previous data from our group demonstrated that elevated ROS in the rostral ventrolateral medulla (RVLM), the site of presympathetic neurons projecting to the spinal cord,5 contributes to sympathoexcitation in a rabbit model of chronic heart failure.6 In addition, redox imbalance in the RVLM has also been suggested to underlie the sympathoexcitation associated with hypertension.7

Antioxidant enzyme activity is the principal endogenous defense mechanism protecting cells and organs against oxidative damage by converting the highly reactive oxygen molecules (O2·− and H2O2) into inert products (O2 and H2O). In response to an oxidant challenge, antioxidant enzymes are dynamically upregulated at both the transcriptional and translational levels. Nuclear factor erythroid 2–related factor 2 (Nrf2) plays an important role in mediating the transcriptional regulation of many antioxidant enzymes.8 Under basal conditions, Nrf2 is sequestered in the cytoplasm in association with Keap1 (Kelch-like ECH associating protein 1), resulting in degradation of Nrf2 by ubiquitination mediated by the Cullin3-based E3 ubiquitin ligation complex.9,10 In response to various forms of oxidant stress, the cysteine residues of Keap1 are oxidized, and the Nrf2–Keap1 complex is disrupted, facilitating translocation of Nrf2 to the nucleus where it binds to the antioxidant responsive elements in the promoter region of multiple genes, leading to upregulation of a battery of antioxidant enzymes, including superoxide dismutase (SODs), NAD(P)H:quinone oxidoreductase 1 (NQO1), heme oxygenase 1 (HO-1), and catalases.11,12

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Although decreased antioxidant enzymes have been well recognized as a major contributor to the central oxidative stress and sympathetic hyperactivity in heart failure and hypertension, the mechanisms underlying the downregulation of these antioxidant enzymes are largely unknown. Furthermore, the roles of Nrf2 in central redox homeostasis and sympathetic regulation remain to be elucidated. Therefore, in the present study, we used an Nrf2-floxed mouse model to determine whether deletion of the Nrf2 gene specifically in the RVLM alters blood pressure (BP) and sympathetic outflow in otherwise normal mice. We hypothesized that mice with impaired Nrf2 signaling in the RVLM will exhibit an increase in sympathetic tone and BP.

**Materials and Methods**

Forty-nine Nrf2-floxed (Nrf2<sup>flox/flox</sup>) mice (28 males and 21 females) aged between 16 and 20 weeks were used in these experiments. Mice were originally obtained from Dr. Shyam Biswal at the Johns Hopkins University. All experiments were approved by the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center and were performed under the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

**Induction of RVLM-Specific Nrf2 Deficiency**

The Nrf2 gene of the Nrf2<sup>flox/flox</sup> mice was modified by inserting 2 loxP sites flanking Exon 5. Excision was accomplished by microinjection of lentiviral Cre recombinase, thus, generating a Nrf2 deficiency model. To selectively delete the Nrf2 gene in the RVLM, lentiviral-Cre-GFP (lentiviral-GFP as the control; 1×10<sup>8</sup> TU/mL, 20 nL; Kerafast, Inc, Boston, MA) was injected bilaterally into the RVLM of Nrf2<sup>flox/flox</sup> mice (referred to as Cre mice and GFP mice) using a mouse stereotaxic instrument (SR-5M-HT; NARISHIGE International USA, Inc, Amityville, NY) under isoflurane anesthesia (<2%) and supplemented with humidified chamber at 37°C for 20 minutes of stable baseline hemodynamics. Mice were then euthanized, and the maximum RSNA was obtained within 1 to 2 minutes, followed by a recording of background noise +15 to 20 minutes after death. Baseline RSNA was determined as the percent of maximum RSNA activity after the background noise was subtracted.

The induced arterial baroreflex sensitivity (IBRS) was analyzed by logistic regression over the entire pressure range after phenylephrine administration. The values of BP and RSNA were acquired every 2 seconds from the threshold to the saturation points. A sigmoid logistic regression curve was fit to the data points using the following equation: RSNA<sub>max</sub> = A<sub>1</sub> / [1 + exp(B (MAP–C))] + D, where A is the RSNA range, B is the slope coefficient, MAP is the mean arterial pressure, C is the pressure at the midpoint of the range (BP), and D is the minimum RSNA. The peak slope (or maximum gain) was determined by taking the first derivative of the baroreflex curve and was calculated with the equation: Gain max = A<sub>1</sub> (1 + A<sub>2</sub>) [-1/4], where A<sub>1</sub> is the range and A<sub>2</sub> is the average slope. The mean values for each curve parameter were used to derive composite curves for each experimental group.

**Biochemical and Molecular Measurements**

**Urinary NE**

To collect urine, mice were placed in a metabolic cage (Harvard Apparatus, Holliston, MA). Daily food/water intake and urine/fecal excretion were measured.

Urinary NE was measured using a Norepinephrine Enzyme Immunoassay kit (Labor Diagnostika Nord KG, Nordhorn, Germany). A 50-μL urine sample was diluted with 950 μL double-distilled H<sub>2</sub>O to obtain a 20:1 diluted sample, from which 10 μL was used for NE measurements based on the instructions provided by the company. Duplicate measurements were made for each sample. Twenty-four-hour NE excretion was calculated by multiplying NE concentration by 24-hour urine volume.

**Evaluation of Oxidative Stress**

1. Dihydroethidium (DHE) staining: The unfixed frozen mouse brain stem was cut into 30-μm sections and placed on glass slides, which were immersed in 2×10<sup>4</sup> mol/L DHE diluted with DMSO and acetone. After incubation in a light-protected, humidified chamber at 37°C for 30 minutes, extra staining solution was removed, followed by 3 rinses with PBS. The fluorescence generated by oxidized DHE was detected using a laser confocal microscope (Leica TSC STED) using 488-nm wavelengths.
excitation wavelength and a 585-nm filter. The relative fluorescent intensity of images was quantified using Image-J software (National Institutes of Health).

2. Electron Paramagnetic Resonance (EPR) Spectroscopy: To obtain ample amount of tissue for EPR spectroscopy, 8 RVLM punches from 4 GFP mice or Cre mice were pooled into a single EPR sample. Immediately after sample collection, RVLM punches were incubated with a superoxide-sensitive EPR spin probe, 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine, for 60 minutes at 37°C in EPR incubation buffer consisting of the following (in mmol/L): 99 NaCl, 4.69 KCl, 2.5 CaCl₂, 1.2 MgSO₄, 25 NaHCO₃, 1.03 KH₂PO₄, 5.6 D-glucose, 20 HEPES and supplemented with the metal chelators diethylthiocarbamate (5 μmol/L) and defereroxamine (25 μmol/L). Samples were then loaded into a 1 mL syringe and flash frozen between EPR buffer solutions to form a continuous frozen plug using liquid nitrogen. The frozen EPR sample plug was then placed into a liquid nitrogen finger dewar and inserted into a Bruker eScan EPR Spectrometer. The following EPR spectrometer settings were used: field sweep width, 100.0 G; microwave frequency, 9.75 kHz; microwave power, 1.10 mW; modulation amplitude, 5.94 G; conversion time, 10.24 ms; time constant, 40.96 ms. It should be noted that the EPR spectrum obtained from the GFP and Cre mice RVLM samples were normalized to the total weight (mg) of tissue in each sample.

**mRNA and Protein Expression**

1. Real-time reverse transcriptase polymerase chain reaction: Total RNA was extracted from the RVLM punches (coordinates: 1.25–1.75 mm lateral to the midline, 1.25–2.00 mm ventral to the dorsal surface of the brain stem, 1.16–1.52 mm cranial from the obex) with TRIZOL reagent (Invitrogen), which was then treated with RNase-free DNase (Invitrogen) and quantified using a Bio-Rad Laboratories microplate spectrophotometer. Total RNA (2 μg) was reverse transcribed into cDNA using a Superscript II reverse transcriptase (Invitrogen) and oligo dT primer (Invitrogen), which was then amplified using a primerset (Table 1). Amplification of β-actin was performed as an internal control. Amplification products were separated by agarose gel electrophoresis, visualized, and transferred to a nylon membrane with a transfer station (Stratagene). Membranes were incubated with blocking solution (TBS, 5% nonfat milk, 0.2% Tween 20) and then with primary antibodies for 2 hours at room temperature. The membranes were washed with TBS containing 0.1% Tween 20 and incubated with horseradish peroxidase-conjugated secondary antibodies (goat anti-rabbit IgG secondary antibody) at room temperature for 2 hours. The blots were developed by chemiluminescence substrate (Pierce) and visualized using a UVP BioImaging System. The final reported data are the target protein band density divided by the GAPDH density.

2. Western Blotting: The target proteins and their primary antibodies were Nrf2 (ab31163 R-IgG), NQO1 (sc-376023 M-IgG), HO-1 (sc-1789 R-IgG), SOD2 (sc-30080 R-IgG), and catalase (sc-34280 G-IgG). GAPDH (sc-32233 M-IgG) served as an internal control. RVLM punches were homogenized in radioimmunoprecipitation assay buffer, and total protein was extracted from the homogenates. Protein concentration was measured using a protein assay kit and then adjusted by adding 4% sodium dodecyl sulfate sample buffer to obtain equal concentrations among these samples. The samples were then loaded on a 10% SDS-PAGE gel (30 μg protein per well) and subjected to electrophoresis. The fractionated protein on the gel was electrophoretically transferred onto a polyvinyl difluoride membrane. The membrane was first probed with the primary antibody to the target protein and then with GAPDH primary antibody. After incubation with primary antibodies, the membranes were probed with secondary antibodies followed by treatment with enhanced chemiluminescence substrate (Pierce). The bands on the membrane were visualized and analyzed using a UVP BioImaging System. The final reported data are the target protein band densities divided by the GAPDH density.

3. Immunofluorescence staining. The area of Nrf2 deletion in the Cre mouse RVLM was confirmed using immunofluorescence staining. Mice were deeply anesthetized with sodium pentobarbital and perfused transcardially with PBS, followed by perfusion of 4% paraformaldehyde in PBS. The entire brain stem was removed, mounted on a specimen stage, and sectioned into 40-μm slices in a cryostat. The slices were then washed with PBS 3× and permeabilized for 30 minutes at room temperature with a solution containing 0.3% Triton X-100 dissolved in PBS, followed by blocking with solution containing 10% normal goat serum and 0.3% Triton X-100 in PBS at room temperature for 2 hours. The slices were then incubated with Nrf2 antibody (ab31163 R-IgG) in 10% normal goat serum and 0.3% Triton X-100 in PBS at 4°C overnight. After 3 washes with PBS, the slices were incubated for 2 hours with secondary fluorescent antibody (goat anti-rabbit IgG secondary antibody, Alexa Fluor 546; Invitrogen, A-11010). The slices were mounted with an Aqua-Mount Mounting Medium and then were examined with a laser confocal microscope (Leica TSC STED).

**Statistical Analyses**

All of the data are expressed as mean±SE. Student’s t test was used to compare the difference between 2 groups, with the aid of SigmaPlot software. A P value of <0.05 was taken as indicative of statistical significance.

**Results**

**Nrf2 mRNA and Protein Expression**

Nrf2 mRNA and protein expression in the RVLM of Nrf2<sup>lox/lox</sup> mice 2 to 3 weeks after bilateral microinjection of lentiviral-GFP (GFP) and lentiviral-Cre-GFP (Cre) was determined (Figure 1A and 1B). Compared with GFP mice, Cre mice exhibited significantly lower Nrf2 mRNA (Figure 1A, 0.30±0.08 versus 1.00±0.11; **P<0.01, n=7) and protein (Figure 1B, 0.07±0.02 versus 0.32±0.03; **P<0.01, n=7) in RVLM punches. These data indicate a transcriptional and translational downregulation of Nrf2 in the RVLM of Cre mice, suggesting that Cre recombinase excised the Nrf2 gene from the genome, leading to a decrease in Nrf2 mRNA, followed by a decreased translation of Nrf2 protein. Although Nrf2 mRNA and protein were not reduced to zero, we think the traces of Nrf2 mRNA and protein may be because of contamination in the vicinity of the RVLM punches rather than incomplete knockout of the Nrf2 gene within RVLM.

Figure 1C shows immunofluorescence images of Nrf2 protein in one brain stem section at the RVLM level of a Nrf2<sup>lox/lox</sup> mouse whose right RVLM was injected with lentiviral-Cre-GFP, and left RVLM was intact as the control. The area of injection into the RVLM shows a strong GFP signal; however, low or absence of a red Nrf2 immuno-positive signal suggest- ing selective deletion of Nrf2 in the neurons infected by lentiviral-Cre-GFP. Importantly, we could find no evidence of GFP staining in other areas of the brain stem and hypothalamus. Therefore, we think that these injections selectively deleted the Nrf2 gene in the RVLM.

**Expressions of Nrf2 Downstream Target Proteins**

Western blot data (Figure 2) shows protein expression of Nrf2 downstream targets, the antioxidant enzymes, in RVLM punches of GFP mice and Cre mice. NQO1, HO1, SOD2, and catalase were all reduced in the tissue from Cre mice compared with the GFP mice (NQO1: 0.37±0.08 versus 1.12±0.10, **P<0.01; HO1: 0.33±0.10 versus 0.90±0.12, **P<0.01; SOD2: 0.52±0.12 versus 0.92±0.11, *P<0.05; catalase: 0.66±0.12 versus 1.27±0.13, *P<0.05; n=7 in each group).
suggested that Nrf2 gene deletion also leads to a downregulation of a battery of antioxidant enzymes.

**Oxidative Stress Measurements**

Figure 3 shows the superoxide level in the RVLM of GFP mice and Cre mice. Figure 3A represents DHE staining showing a stronger red fluorescent signal and higher quantified density of red fluorescence in RVLM of Cre mice as compared with GFP mice (71.8±3.0 versus 39.2±1.7, *P<0.05, compared with GFP mice; n=6 for each group). Figure 3B shows EPR spectra obtained from fresh RVLM punches pooled from 4 GFP mice and 4 Cre mice after incubation with the superoxide-sensitive EPR spin probe, 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine HCl. On reacting with superoxide, the 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine spin probe becomes a stable nitroxide radical, which when placed in the appropriate magnetic field scan (Gauss) yields the characteristic spectra shown in Figure 3B.21 Notably, each spectrum was normalized to the total weight (milligram) of tissue in the respective sample. Considering the amplitude of the EPR spectrum is directly proportional to the levels of superoxide in the sample,21 the enhanced EPR spectrum amplitude obtained from Cre mice RVLM punches compared with that from GFP mice RVLM punches indicates an increase in superoxide levels in the RVLM of Cre mice. Collectively, these data suggest that in the RVLM, Nrf2 deletion results in an increase in ROS, particularly superoxide.

**Cardiovascular Sympathetic Regulation in the Conscious State**

Measurements of BP and sympathetic tone were performed to assess the systemic effects of RVLM Nrf2 deletion. Figure 4A shows BP and HR data grouped by 1-hour averages (left) and 24-hour average data (right). GFP mice and Cre mice exhibited...
a similar circadian rhythm of BP and HR. Blood pressure in Cre mice was elevated in both nighttime and daytime compared with that in GFP mice (24-hour average mean arterial pressure: 123.7±3.8 versus 100.2±2.2 mm Hg, *P<0.05, n=6), whereas there was no difference in HR between these 2 groups (24-hour average HR: 531.2±13.9 versus 536.1±14.3 bpm, n=6). No differences in BP or HR response were observed between male and female mice (data not shown). Figure 4B shows the spontaneous arterial baroreflex sensitivity evaluated in the conscious state. Both up-sequence gain (1.66±0.17 versus 3.61±0.22 ms/mm Hg, *P<0.05, n=6) and down-sequence gain (1.89±0.12 versus 2.98±0.19 ms/mm Hg, *P<0.05, n=6) were significantly decreased in Cre mice compared with the GFP mice, suggesting impaired baroreflex function in mice with Nrf2 deletion in the RVLM. In addition, both urinary NE concentration (456.4±16.9 versus 356.5±19.9 ng/mL, *P<0.05; n=13–14) and 24-hour NE excretion (676.5±64.2 versus 416.5±28.1 ng/24 h, *P<0.05; n=13–14) were higher in Cre mice, suggesting a sympathoexcitatory effect of RVLM Nrf2 knockdown in the Cre mice. However, there was no difference in water/food intake or urine/fecal excretion between these 2 groups (data not shown).

Cardiovascular and Sympathetic Regulation in the Anesthesia State

Figure 5 shows the RSNA and IBRS in GFP mice and Cre mice in the anesthetized state. Baseline BP, HR, and RSNA are shown in Figure 5A (top, original recording; bottom, mean data). Cre mice exhibited significantly higher baseline RSNA as compared with the GFP mice (63.8±8.1 versus 24.2±4.3% of max, **P<0.01 versus GFP mice, n=6 for each group). Figure 5B shows arterial baroreflex data original recording (left, top), composite baroreflex curves (right, top; inset is the gain of IBRS), and the 5 parameters reflecting IBRS sensitivity (bottom). The representative recording shows transient silencing of RSNA and a profound decline of HR when the BP was increased by phenylephrine administration in a GFP mouse, whereas the decrease of RSNA and HR to the phenylephrine -evoked hypotension was markedly blunted in Cre mice, suggesting an impairment of baroreflex function in mice with Nrf2 deletion in the RVLM. Indeed, the bottom graphs of Figure 5B show that the average slope (0.083±0.01 versus 0.119±0.01% of mm Hg, **P<0.01), maximal gain (1.64±0.20 versus 2.66±0.20% of mm Hg, **P<0.01), and range of RSNA response of baroreflex (69.2±6.8 versus 89.4±8.4% of max; *P<0.05) were significantly decreased, whereas the BP50 (102.3±8.9 versus 80.1±11.5 mm Hg; *P<0.05) and minimum RSNA of baroreflex (23.3±2.5 versus 9.0±1.2% of max; *P<0.05) were significantly higher in Cre mice compared with GFP mice (n=6 for each group).

Discussion

Redox homeostasis is an essential function maintaining the milieu intérieur in the physiological state. It has been well recognized that central oxidative stress plays a crucial role in maladaptive cardiovascular function and sympathetic outflow in chronic heart failure and hypertension. Excessive central, especially brain stem and hypothalamic, ROS in cardiovascular disease is derived from an imbalance between pro-oxidative and antioxidant processes. This imbalance results in intracellular and perhaps extracellular ROS accumulation, leading to neural dysfunction. Indeed, in heart failure and hypertension, nicotinamide adenine dinucleotide phosphate oxidase has been shown to be activated and SODs downregulated in key cardiovascular nuclei, such as the RVLM, thus, contributing to sympathoexcitation.

In the present study, we determined whether selective deletion of the antioxidant enzyme transcription factor Nrf2 in the RVLM altered sympathetic nerve activity and blood pressure in normal mice. By using Nrf2flox/flox mice and using lentiviral-Cre-GFP microinjection, we successfully deleted the Nrf2 gene in the RVLM, resulting in a decrease in Nrf2 mRNA and protein expression, which lead to a downregulation of antioxidant enzymes and elevation of ROS. The most profound finding in this study was a significant elevation of blood pressure associated with an increase in urinary NE concentration and excretion, elevated RSNA, and a blunted arterial baroreflex control of HR in the conscious state (spontaneous baroreflex sensitivity) and suppression of sympathetic nerve activity in the anesthetized state (IBRS). These findings strongly suggest that in the RVLM of normal mice, Nrf2 gene deficiency contributes to ROS generation, sympathoexcitation, and hypertension.

The RVLM is considered to be one of the major brain stem nuclei where presympathetic neurons project to the spinal cord and regulates sympathetic outflow and, thus, cardiovascular...
function. In addition, projections from other mid-brain, hypothalamic, and brain stem nuclei such as subfornical organ, hypothalamic paraventricular nucleus, and median preoptic nucleus, converge on the RVLM where projections to the intermediolateral cell columns of the spinal cord providing sympathetic preganglionic neurons to the periphery. The

Figure 4. Blood pressure and heart rate (HR, A), spontaneous arterial baroreflex sensitivity (B), and urinary norepinephrine (NE) excretion (C) in conscious mice with bilateral microinjection of lentiviral-GFP (GFP) or lentiviral-Cre-GFP (Cre). *P<0.05 compared with GFP mice, n=6 for each group. MAP indicates mean arterial pressure.

Figure 5. Baseline renal sympathetic nerve activity (RSNA, A) and induced arterial baroreflex sensitivity (IBRS, B) in anesthetized mice with bilateral microinjection of lentiviral-GFP (GFP) or lentiviral-Cre-GFP (Cre). *P<0.05 and **P<0.01 compared with GFP mice, n=6 for each group. BP indicates blood pressure.
RVLM also receives inhibitory input originating from peripheral reflexes and projections from the nucleus tractus solitarius and caudal ventrolateral medulla, thus, modulating the arterial baroreflex regulation on HR and sympathetic nerve activity.31

Neurons in the RVLM are susceptible to increased ROS. Sympathoexcitation in several rat models of hypertension has been attributed to oxidative stress in the RVLM.30 Indeed, in spontaneous hypertensive rats (both spontaneously hypertensive rats and spontaneously hypertensive stroke prone rats)12,33 and in secondary hypertension (high salt-, obesity-, and jet leg–induced),34–36 superoxide levels in the RVLM are increased, contributing to sympathoexcitation and the elevated blood pressure. In previous studies from our laboratory, we showed increased oxidative stress in the RVLM in rabbits with heart failure that was responsible for augmented sympathoexcitation.5,25,37 The mechanism(s) by which this increased sympathoexcitation takes place has not been completely defined.

The present data showing sympathoexcitation and hypertension in mice after Nrf2 knockdown in the RVLM suggests a tonic production of ROS in this critical sympathetic nucleus under physiological conditions and that oxidant stress can evoke cardiovascular and sympathetic dysfunction of a sustained nature. The source of ROS in the RVLM is not completely clear. Potential pro-oxidative mechanisms in brain include nicotinamide adenine dinucleotide phosphate oxidase, xanthine oxidase, uncoupled nitric oxide synthase, and mitochondria.38–40 NAD(P)H oxidase is a major contributor to superoxide generation in the RVLM, especially in response to activation of the angiotensin II–angiotensin type 1 receptor signaling pathway, which is activated in hypertension and chronic heart failure.6,41 It is not clear if a presynaptic or postsynaptic mechanism or both mediate ROS modulation of sympathetic nerve activity in the RVLM. In the RVLM of SHR, it has been demonstrated that ROS improved the release of the excitatory amino acid glutamate, while suppressed the release of the inhibitory amino acid GABA.33 On the other hand, in rats with coronary artery ligation–induced heart failure, we documented that increased superoxide mediated angiotensin II–induced downregulation of the potassium channel protein, Kv4.3 expression, and increase in neuronal excitability in the RVLM.42 In dissociated neurons from rat hypothalamus and brain stem, angiotensin II–induced ROS decreased potassium channel opening, leading to an inhibition of a delayed rectifying potassium current and membrane depolarization.43 These studies suggest that both presynaptic and postsynaptic mechanisms are responsible for ROS neuronal modulation in the RVLM to induce sympathoexcitation. Glia are another potential source of ROS in the RVLM. Recently, cross talk between glial cells and presympathetic neurons within RVLM and sympathetic tone have been demonstrated.44 Although the functional implication of ROS in this process is lacking, the potential involvement of glial cells in the maladaptive sympathetic regulation seen in these Nrf2–deficient mice cannot be excluded. While we did not specifically examine glia in the RVLM in this study, the immunofluorescent images and DHE staining show that RVLM neurons are at least one source of increased ROS after lentiviral-Cre microinjection. All of the RVLM neurons responsible for sympathetic regulation and directly innervating sympathetic preganglionic neurons of intermediolateral are glutaminergic, among which ≈70% neurons also synthesize adrenaline, defined as the C1 group.28,45–48 One limitation of the present study is that we cannot discriminate the neuronal subtypes where Nrf2 was deleted by lentiviral-Cre.

Nrf2 is a master regulator of a battery of antioxidant enzymes and plays a central role in protecting cells against oxidant damage in a variety of organs and tissues, including the central nervous system.49 The significantly lower protein expression of NQO1, HO-1, SOD2, and catalase and elevated ROS level in the RVLM of mice with Nrf2 knockdown in the present study further confirms this concept. Given dynamic and aggressive activation in response to oxidative stress, Nrf2 may represent a critical adaptive mechanism to maintain central redox balance. In neurogenic hypertension or other chronic cardiovascular diseases characterized by sympathoexcitation, the central redox balance shifts to the pro-oxidative side,5,13,37 which, in turn, evokes antioxidant defenses. Several key antioxidant enzymes in the RVLM of these diseases have been documented to be downregulated,49,13,37 suggesting a mal-adaptation of oxidative stress perhaps because of the impaired Nrf2 signaling. Accordingly, it will be important to evaluate Nrf2 expression and to determine whether impaired Nrf2 signaling contributes to the redox imbalance in the RVLM under these pathological conditions. If so, activation of Nrf2 signaling selectively in the RVLM may be a novel therapeutic strategy for hypertension and chronic heart failure. However, it has not been established that NQO1 and HO-1 expressions are altered in the RVLM of hypertensive and chronic heart failure animals. In a recent study by Wu et al.,48 it was shown that a suppressed Nrf2 function in the RVLM of hypertensive rats induced by lipopolysaccharide leads to a defect in mitochondrial biogenesis and contributes to the pathogenesis of hypertension. However, in the current study, it is not clear if mitochondrial function was altered and contributed to the elevated blood pressure in mice with Nrf2 deficiency.

Lenti-Cre treated mice in the current study exhibited reduced arterial baroreflex sensitivity. An appropriate baroreflex depends on the functional integrity of baroreceptor afferents, central neural circuits (nucleus tractus solitarius, caudal ventrolateral medulla, and RVLM), efferent pathways (parasympathetic and sympathetic nerves), and effector organs (heart and blood vessels).40 The blunted baroreflex sensitivity in Nrf2–deficient mice in this study is most likely because of efferent dysfunction originating in the RVLM. We found no GFP fluorescence outside of the RVLM after lenti-Cre microinjection. As indicated earlier, there is support for the notion that increased ROS can enhance glutamate release and reduce GABA release.33 The current data does not allow us to establish a causal relationship between baroreflex dysfunction, sympathoexcitation, and hypertension in mice where Nrf2 has been knocked down in the RVLM. However, it is clear that the hypertension in these mice was associated with an increase in sympathetic outflow. Interestingly, there was no change in HR after lenti-Cre administration. While it is not clear why HR did not increase as did BP, it may be that the high level of cardiac sympathetic tone in mice represents a ceiling effect so that further increases are unlikely. Indeed, cardiac sympathetic tone in mice is significantly higher compared with that in rats, whereas vascular sympathetic tone is similar in these
2 species. Given that mice have a limited reserve to increase cardiac output, vasconstriction-induced increase in peripheral resistance is likely the predominant contributor to the hypertension seen in these RVLM Nrf2 knockout mice.

In summary, we show here that selective deletion of the Nrf2 gene in the RVLM of mice results in a remarkable decrease in Nrf2 mRNA and protein expression, leading to a downregulation of HO-1, NQO1, SOD2, and catalase protein expression and an elevation of superoxide and ROS in the RVLM. This mouse model displays sustained hypertension, sympathoexcitation, and baroreflex dysfunction. These data suggest that Nrf2 plays a critical role in the modulation of redox homeostasis in the RVLM, contributing to cardiovascular function and sympathetic regulation.

**Perspectives**

Essential hypertension is a sympathoexcitatory state that can be difficult to treat. In many cases, patients are resistant to pharmacological therapy. Therefore, a search for novel mechanisms that regulate sympathetic nerve activity is of importance. The study reported in this article focused on the regulation of central antioxidant enzyme expression in normotensive mice after deletion of the Nrf2 gene and protein in the RVLM. The experiments resulted in sympathoexcitation and hypertension. Because oxidative stress is an important regulator of neuronal activity, activation of Nrf2/antioxidant signaling in the brain may be a novel target for therapy in hypertension and other sympathoexcitatory states.

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**Disclosures**

None.

**References**


Novelty and Significance

What Is New?

• For the first-time excision of the nuclear factor erythroid 2–related factor 2 (Nrf2) gene in the rostral ventrolateral medulla of normal mice results in chronic hypertension.
• A novel use of the nuclear factor erythroid 2–related factor 2 floxed mouse is reported.

What Is Relevant?

• Hypertension is, in part, mediated by sympathoexcitation.
• Adaptive molecular mechanisms that target oxidative stress in the brain may be important therapeutic targets.

Summary

These data clearly indicate that targeted deletion of nuclear factor erythroid 2–related factor 2, a master regulator of antioxidant gene transcription, increases oxidative stress in the rostral ventrolateral medulla of normal mice, resulting in a decrease in antioxidant gene expression, an increase in sympathetic activation, a decrease in baroreflex sensitivity, and sustained hypertension of a magnitude equivalent to chronic infusion of pressor agents (eg, angiotensin II).
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Lie Gao, Matthew C. Zimmerman, Shyam Biswal and Irving H. Zucker

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