THE NRF2 KNOCKOUT RAT: A NEW ANIMAL MODEL
TO STUDY ENDOTHELIAL DYSFUNCTION, OXIDANT STRESS, AND
MICROVASCULAR RAREFACTION

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microvessel density experiments, wrote initial versions of manuscript with JRCP, edited
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Short Title: Development of a Nrf2(-/-) Mutant Rat

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ABSTRACT

Nuclear factor (erythroid-derived 2)-like-2 (NRF2) is a master antioxidant and cell protective transcription factor that upregulates antioxidant defenses. In this study we developed a strain of Nrf2 null mutant rats to evaluate the role of reduced NRF2-regulated antioxidant defenses in contributing to endothelial dysfunction and impaired angiogenic responses during salt-induced ANG II suppression. Nrf2\(^{(-/-)}\) mutant rats were developed using TALEN technology in the Sprague-Dawley genetic background, and exhibited a 41 base pair deletion that included the start codon for Nrf2 and an absence of immunohistochemically-detectable NRF2 protein. Expression of mRNA for the NRF2-regulated indicator enzymes hemeoxygenase-1, catalase, superoxide dismutase 1, superoxide dismutase 2, and glutathione reductase was significantly lower in livers of Nrf2\(^{(-/-)}\) mutant rats fed high salt (HS; 4% NaCl) for two weeks compared to wild type controls. Endothelium-dependent dilation to acetylcholine (ACh) was similar in isolated middle cerebral arteries (MCA) of Nrf2\(^{(-/-)}\) mutant rats and wild type littermates fed low salt (0.4% NaCl) diet, and was eliminated by short term (3 days) HS diet in both strains. Low dose ANG II infusion (100 ng/kg s.c.) reversed salt-induced endothelial dysfunction in MCA and prevented microvessel rarefaction in wild type rats fed HS diet, but not in Nrf2\(^{(-/-)}\) mutant rats. The results of this study indicate that suppression of NRF2 antioxidant defenses plays an essential role in the development of salt-induced oxidant stress, endothelial dysfunction, and microvessel rarefaction in normotensive rats; and emphasizes the potential therapeutic benefits of directly upregulating NRF2-mediated antioxidant defenses to ameliorate vascular oxidant stress in humans.
**Key Words:** Nuclear factor (erythroid-derived 2)-like-2 (NRF2); Angiotensin II; Oxidant Stress; Endothelium; Salt; Nitric Oxide; Angiogenesis

**New and Noteworthy:** A novel rat strain with a null mutation of the gene coding for the master antioxidant transcription factor NRF2 has been developed. The paradoxical protective effect of low dose angiotensin II infusion to ameliorate endothelial dysfunction and prevent microvascular rarefaction in salt-fed animals is absent in Nrf2(-/-) mutant rats.
INTRODUCTION

A state of redox homeostasis is critical to maintaining normal vascular function. Elevated levels of reactive oxygen species (ROS) not only reduce nitric oxide availability, but also generate reactive nitrogen species such as peroxynitrite, which directly impairs signaling pathways mediating vascular relaxation (2, 3, 22, 26). In addition to its effects on the vasculature, increased oxidant stress has deleterious effects on multiple other organs and tissues including lung (5, 35, 48, 49, 55), liver (44, 61), and brain (1, 53, 54, 63), emphasizing the importance of antioxidant defense systems throughout the body.

Nuclear factor (erythroid-derived 2)-like-2 (NRF2 or NFE2L2) is a redox sensitive transcription factor that binds to the antioxidant response element (ARE) in the promoter region of hundreds of antioxidant and cytoprotective genes. (21, 23) Together with its cytosolic inhibitor protein Kelch-like ECH-associated protein 1 (Keap1) (24), NRF2 senses oxidant/electrophilic stress and coordinates a transcriptional defense response. Recently, NRF2 has emerged as an attractive research target because dysregulation of NRF2 may provide a logical explanation for the association between oxidant stress and more than 200 human diseases (21).

Although Nrf2 knockout mice exist, the development of a Nrf2(-/-) mutant rat is significant and important because there is an extensive infrastructure of genetic and physiological data on the rat; the rat is better suited for physiological studies than the mouse because of its larger body size; and the rat is the preferred model within the pharmaceutical industry (25). An additional advantage of using TALEN methodology or other gene editing approaches to delete the Nrf2(-/-) gene in the rat is the availability of
multiple specialized rat strains sensitized to model various human diseases, such as low-renin salt-sensitive hypertension (Dahl salt-sensitive rat), pulmonary hypertension [Fawn-hooded Hypertensive rat (FHH)], metabolic syndrome (obese Zucker rat and JCR rat), and non-obese Type 2 diabetes mellitus [Goto-Kakizaki (GK) rat] that are not available in the mouse. The latter possibility could open new avenues of discovery regarding the role of NRF2 antioxidant defenses in various pathological conditions designed to mimic human disease.

High salt (HS) diet suppresses renin release and reduces plasma angiotensin II (ANG II) levels. Congruent with studies in healthy human volunteers demonstrating endothelial dysfunction after short-term salt loading (58), elevated dietary salt intake in normotensive animals rapidly results in endothelial dysfunction (33, 46, 56), oxidant stress (31, 46, 65), suppressed antioxidant enzyme expression/activity (29), enhanced pro-oxidant enzyme activity (30), and impaired endothelial Ca\(^{2+}\) signaling (64).

In animal models, salt-induced vascular dysfunction can be prevented by acute scavenging of reactive oxygen species (46) or, paradoxically, via intravenous infusion of a subpressor dose (3-5 ng/kg/min) of ANG II to restore physiological levels of ANG II in the blood (9, 10, 37, 38). Consistent with studies showing beneficial effects of low dose ANG II infusion on collateral vessel development following coronary occlusion in WKY rats (50), low dose ANG II infusion also prevents salt-induced microvascular rarefaction in HS-fed Sprague-Dawley rats (18) and impaired angiogenic responses to muscle stimulation in HS-fed SS.13\(^{BN}\) consomic rats carrying a normally functioning renin gene in the Dahl salt-sensitive genetic background (8). A potential common denominator in all those effects of ANG II infusion is the recovery of vascular redox homeostasis.
However, the relationships between HS diet, physiological ANG II levels, and the ARE-NRF2-KEAP1 axis have not been investigated.

In the present study, we developed a novel rat strain with a deletion of the *Nrf2* gene to test the hypothesis that the NRF2 antioxidant defense system plays an important role in endothelial dysfunction and microvascular rarefaction associated with elevated dietary salt intake. An additional goal of these experiments was to utilize this novel rat strain to assess the potential role of NRF2 in mediating the protective effect of chronic low dose ANG II infusion [to prevent salt-induced ANG II suppression (15, 64)] in ameliorating endothelial dysfunction and preventing microvascular rarefaction in rats fed high salt diet.

**METHODS**

**Animals:** Initial experiments evaluating the expression of NRF2-regulated indicator enzymes in *Nrf2*\(^{-/-}\) mutant rats and wild type littermates utilized 8-10 week old male rats (see description below) that were maintained on low salt (LS, 0.4% NaCl) diet or switched to a high salt (HS; 4.0% NaCl) diet for 2 weeks. To evaluate the role of NRF2 in mediating the protective effect of chronic low dose ANG II infusion to restore endothelial function (33, 37, 38, 46, 60, 64, 65) and prevent microvascular rarefaction (16, 18), a second group of animals (short term HS diet) was switched to HS diet for three days prior to receiving a subcutaneous osmotic minipump containing either isotonic saline or ANG II for an additional three days, as previously described (9, 46).

In preliminary experiments (not shown), we determined that maximal restoration of vasodilator responses to acetylcholine (ACh), the stable prostacyclin analogue iloprost, the G-protein activator cholera toxin, and reduction of perfusate/superfusate PO\(_2\) to ~40 torr in middle cerebral arteries (MCA) of HS-fed Sprague-Dawley (S-D)
parental rats occurred during s.c. infusion of 100 ng/kg/min ANG II, while infusion of
lower doses (25 ng/kg/min; 50 ng/kg/min) or higher doses of ANG II (200 ng/kg/min,
1000 g/kg/min) was less effective (or ineffective) in restoring vascular relaxation in
response to these vasodilator stimuli, all of which were eliminated by HS diet. Based on
those results, the 100 ng/kg/min dose was used in the present experiments. That dose
of ANG II also prevented salt-induced down regulation of Cu/Zn superoxide dismutase
(SOD1) expression in cerebral arteries, mesenteric resistance arteries, and aortas of S-
D rats fed HS diet (not shown). All animal protocols were approved by the Medical
College of Wisconsin IACUC.

**Generation of Nrf2(-/-) Mutant Rats:** The *SD-Nfe212em1Mcwi* mutant rat (hereafter
called the *Nrf2(-/-) mutant*) developed for these studies was created on the Sprague-
Dawley (S-D) genetic background utilizing transcription activator-like effector nuclease
(TALEN) technology. *In vitro*-transcribed messenger RNAs encoding FLASH XTN™
TALENs targeting the sequence:

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TAGTCCGTGCGGTGGCAATTCCAAGTCCATCATGCTGAGGGCGGACGCTGCGCTA
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within the first exon of the rat *Nrf2* gene were obtained from Transposagen
Biopharmaceuticals, Inc. and injected (10 ng/µL) into Sprague-Dawley (Crl:SD, Charles
River Laboratories) embryos as previously described (12). Embryos were implanted in
pseudopregnant females, and pups were genotyped using the following primers: 5’-
TCCGCTTTTAAGTTCTTGCTCCGT-3’ (forward), 5’-AAGCCGGAGCTTCTTCTACCC
3’ (reverse). A candidate mutant founder was identified using the Surveyor™ Nuclease
Assay (Transgenomic, Inc.) (12) and backcrossed to the S-D strain to establish a
heterozygous mutant colony. Sanger sequencing confirmed germ line transmission of a 41 bp deletion
(CTCAGC\textbf{ATG}ATGGACTTGGAATTGCCACCGCCAGGACTACA) within the first exon, eliminating the Nrf2 translation start codon (underlined and bolded). Experimental homozygous mutant and control sibling wild type (WT) animals were generated for all studies by intercrossing heterozygotes and were genotyped using a simple sequence length polymorphism (SSLP) genotyping protocol on the Applied Biosystems 3730xl DNA Analyzer, where the forward primer above was tagged with an M13 tail (41).

**Verification of Nrf2 Mutation via Polymerase Chain Reaction and Immunohistochemistry:** To verify the mutation via PCR studies, total RNA was extracted from 25 mg kidney and liver samples from Nrf2 mutant and WT littermates using a commercially available kit (IBI Scientific). RNA was reversed transcribed to cDNA using an iScript™ cDNA synthesis kit (BioRad). The cDNA was amplified via PCR with Platinum Taq Polymerase (Life Technologies) using the following primer sequences: 5'-CTATCTGCTGGTTCCCCACTGCT-3' (forward) and 5'-GGCTGGCTGAATTGGGAGGA-3' (reverse) to amplify the region targeted by the TALEN sequence. The thermal profile was 95°C denaturation for 5 minutes, 32 cycles of 95°C for 30 seconds, 61°C for 30 seconds, and 68°C for 40 seconds; followed by 5 minutes of extension at 68°C. Amplification products were run on a pre-cast 10% agarose gel (Bio-Rad), stained for 5-10 minutes with ethidium bromide and were imaged with ultraviolet light.

Successful knockout of NRF2 protein was obtained via immunohistochemistry in kidney slices from WT and Nrf2^{(-/-)} mutant rats. For those studies, kidneys were obtained
from Nrf2(-/-) mutant and WT rats maintained on LS diet. The kidneys were paraffin-
embedded before transverse sectioning into 4 μm thick slices. After de-paraffinizing the
samples, a standard immunohistochemistry protocol (40) was followed using dilutions of
1:50 anti-NRF2 primary antibody (Santa Cruz) and 1:100 horseradish peroxidase-
conjugated anti-rabbit secondary antibody. Samples were viewed using the bright field
filter on a Nikon Eclipse 80i microscope and pictures were taken with a Nikon DS-Fi1
camera.

**Evaluation of NRF2-Regulated Indicator Enzymes via Real Time PCR:** To
evaluate the effect of the Nrf2 mutation on specific indicator enzymes known to be
regulated by NRF2, we obtained 90-100 mg tissue samples from the liver of HS-fed
Nrf2(-/-) mutant rats and wild type littermates fed HS diet for 2 weeks. RNA was isolated
utilizing a TRlzol procedure according to the manufacturer’s instructions (Life
Technologies). cDNA was synthesized using an iScript cDNA synthesis kit (BioRad).
Real-time quantitative PCR was carried out on an Applied Biosystems ABI Prism qPCR
system utilizing RT2 SYBR Green ROX qPCR Mastermix (Qiagen) with the following
thermal profile: 50º C for 2 minutes; 95º C for 10 minutes; followed by 95º C for 15
seconds and 60º C for 60 seconds for 40 cycles. Expression of mRNA for the NRF2
regulated indicator enzymes heme oxygenase-1 (HO-1), superoxide dismutase 1
(SOD1), superoxide dismutase 2 (SOD2), catalase, and glutathione reductase (GSR),
was evaluated utilizing the following primer sequences: 1) HO-1: **Forward 5’-3’:**
aagaggctaagaccgccttc; **Reverse 5’-3’:** cctctggcgaagaaactctg; 2) SOD1: **Forward 5’-3’:**
cgtcattcacttcgagcaga; **Reverse 5’-3’:** attgtccccatattgatgga; 3) SOD2: **Forward 5’-3’:**
gctggcttgcttcataag; **Reverse 5’-3’:** acacatcaatccccagcagt; 4) Catalase: **Forward 5’-
3': tgcacagagccggatctc; Reverse 5'-3': ctcacacaccttgcttgg; and 5) GSR: Forward 5'-3': cgtgcactcggaattcatac; Reverse 5'-3': gtaagcatcccgcttctc. Ribosomal subunit (r18S) primers (Forward 5'-3': tgggcatgattaaggg; Reverse 5'-3': agtcggcattgattagtc) were used as the internal control and the ∆∆CT method was utilized to quantify the respective mRNA levels.

Response to Vasodilators and Microvessel Density Measurements: Middle cerebral arteries (MCA) were isolated, mounted on glass micropipettes pressurized to 80 mm Hg, and maintained at 37°C in bicarbonate-buffered physiological salt solution (PSS) equilibrated with a 21% O₂-5% CO₂- 74% N₂ gas mixture (11, 33, 56). To evaluate the potential role of Nrf2 in mediating the previously described protective effect of low dose angiotensin II (ANG II) infusion to restore endothelium-dependent dilation, responses to increasing concentrations of the endothelium-dependent dilator acetylcholine (ACh) and the NO donor sodium nitroprusside (SNP) (11, 37) were evaluated in MCA of the Nrf2(-/-) mutant rats and WT littermates fed either a low salt (LS) or a short-term (3 days) HS diet. At the end of the experiment, active resting tone (T) was calculated as: T(%)=\[(D_{max}-D_{rest})/D_{max}\] x 100--where D_{max} is the maximum diameter in Ca²⁺-free PSS and D_{rest} is the diameter at the 80 mm Hg equilibration pressure in normal PSS. Vessel responses to ACh were expressed as percent of the maximal dilator capacity of the artery [(diameter increase to ACh/maximum diameter increase in Ca²⁺-free PSS at 80 mm Hg) x 100].

Microvessel density was assessed in the cremaster muscle of Sprague-Dawley (parental strain) rats, Nrf2(-/-) rats, and wild type littermates fed short term HS diet and receiving an infusion of a low dose ANG II or isotonic saline vehicle for 3 days. Vessels
were visualized using rhodamine-labeled *Griffonia simplicifolia*-1 (GS-1) lectin as described previously (13, 17, 18); and the results were quantified by counting the intersections of vessels with a superimposed 19X14 grid generated using Metamorph Imaging software in 60-80 photographs taken in the cremaster muscles of each animal.

**Cultured Endothelial Cell Preparation:** Because translocation to the nucleus represents a critical step in NRF2 activation of antioxidant defense mechanisms, we determined whether ANG II affects the subcellular localization of NRF2 fluorescence in thoracic aortic endothelial cells cultured from male Sprague-Dawley rats fed short term HS diet. In those experiments, the animals were sacrificed and their thoracic aortas were removed and cleaned of adipose and connective tissues using sterile surgical tools. The aortas were cut into many smaller rings and incubated in microcentrifuge tubes containing 0.2% collagenase I (Sigma-Aldrich) solution in Hank’s balanced salt solution (HBSS) for 45 minutes at room temperature. Tubes were centrifuged at 1,000 G for 10 minutes, and the supernatant was transferred to a cell culture flask containing growth medium for colony expansion and passaging. The growth medium used in these experiments was Dulbecco’s Modified Eagle’s medium (DMEM) containing 10% fetal bovine serum, 1% antibiotic-antimycotic (containing 10,000 units/mL of penicillin, 10,000 µg/mL of streptomycin, and 25 µg/mL of Fungizone®, an anti-fungal agent), and 400 µM L-alanyl-L-glutamine (GlutaMAX™). All cell culture reagents were supplied by Life Technologies (Thermo Fisher Scientific), unless otherwise noted. On the second or third day of culture, non-adherent cells were removed and the growth medium was replaced. The medium was changed every 3-4 days until flasks reached confluency, at which point the colonies were passaged. The experiments in this study utilized cells at
passage number 1-2. Growth of an endothelial cell population was confirmed by visually inspecting the colonies weekly for the classic cobblestone morphology characteristic of endothelial cells and by confirming endothelial cell function utilizing a tube-formation assay, as described by Hoffmann et al. (20).

To assess the effect of ANG II on the translocation of NRF2 to the nucleus, cultured aortic endothelial cells were incubated with either saline vehicle or 100 nM ANG II for 24 hours. After the incubation period, the cells were fixed in a 1:1 acetone/methanol solution at -20°C for 20 minutes. Wells were rinsed with Dulbecco's Phosphate-buffered saline (DPBS) and then blocked in blocking buffer (5% goat serum and 0.3% Triton-X 100 in DPBS) for one hour at room temperature. NRF2 was labeled by incubating the coverslips overnight at 4°C with a 1:200 dilution of primary antibody (anti-NRF2 antibody--Santa Cruz Biotechnology Inc. Dallas, TX); rinsed with DPBS, and incubated in the dark for 4 hours at room temperature with a fluorescent-labeled secondary antibody (1:1000 Alexa 488 anti-rabbit) followed by a final rinse in DPBS. The coverslips were then adhered face down on a glass microscope slide using VECTASHIELD mounting medium with DAPI (4,6-diamidino-2-phenylindole) (Vector Laboratories, Burlingame CA)--a fluorescent dye that emits a blue wavelength when bound to double-stranded DNA and is used in immunofluorescence studies as a nuclear marker (27).

Slides were imaged on a Nikon Eclipse 80i microscope (Nikon, Tokyo, Japan) using MetaMorph imaging software (Molecular Devices, Sunnyvale, CA). Cells were located on the slide using the DAPI microscope filter; and green Nrf2 fluorescence was captured from five randomly selected regions. Later, the number of total nuclei imaged
and the number of NRF2-positive nuclei [where DAPI and NRF2 fluorescence co-localized (see representative image in Figure 4)] were manually counted in each image and the results were quantified by expressing the number of NRF2-positive nuclei as a percentage of total nuclei visualized.

**Statistical Methods:** Data are expressed as the mean ± standard error of the mean. Differences between means were evaluated using Student's t-tests, one-way analysis of variance (ANOVA) with a Kruskal-Wallis test post hoc or two-way ANOVAs with a Student-Newman-Keuls test post hoc, as appropriate. A P-value of P<0.05 was considered to be statistically significant.

**RESULTS**

**Effect of Nrf2 Mutation on Baseline Parameters:** The baseline parameters of body weight, mean arterial pressure, resting and maximum MCA diameter, and active tone were unaffected by the Nrf2 mutation, diet, or subcutaneous infusion of saline or ANG II (Table 1). These findings are consistent with previous reports in Nrf2(-/-) mice showing that elimination of Nrf2 does not affect growth or development (6); and also indicate that the Nrf2(-/-) mutation has no effect on active resting tone or structure of the MCA, and does not increase arterial blood pressure under the conditions of our study.

**Verification of the Nrf2 Mutation:** PCR amplification of the region containing the DNA deletion showed the expected full PCR products of 389 bases for the wild type Nrf2 transcript samples, while Nrf2(-/-) mutant samples showed smaller bands at 348 bases, indicative of the 41-base deletion mutation (Figure 1). In the immunohistochemical studies (Figure 2), kidneys from WT rats showed widespread dark-brown staining, indicative of ubiquitous NRF2 protein localization (6, 23) that was absent in kidneys from rats homozygous for the Nrf2 mutation. The absence of NRF2
protein in those structures provided further verification that we had successfully
generated a *Nrf2* null rat. Expression of mRNA for the Nrf2-regulated indicator
enzymes catalase, HO-1, SOD1, SOD2, and GSR was also significantly lower in livers
of *Nrf2*(-/-) rats fed HS diet for 2 weeks compared to those from HS-fed WT littermates
(Figure 3).

**ANG II-Induced Translocation of Nrf2 to the Nucleus of Cultured
Endothelial Cells:** Cultured aortic endothelial cells from Sprague-Dawley rats fed HS
diet showed diffuse fluorescence of Nrf2 in the cytoplasm, consistent with binding of
Nrf2 by KEAP1 anchored to the cytoskeleton. Incubation of the cells with 100 nM
ANG II for 24 hours caused translocation of Nrf2 to the cell nucleus, as indicated by a
shift in Nrf2 fluorescence away from the cytosol and into distinct regions of the nucleus
(Figure 4), and a significant increase in Nrf2-positive nuclei [from 36±9% Nrf2
positive cells with vehicle treatment (n=6) to 72±15% Nrf2 positive cells after 24-hour
incubation with ANG II (n=6)]—P<0.05, Student’s t-test.

**Effect of Low Dose ANG II Infusion on Responses of Middle Cerebral
Arteries to Acetylcholine (ACh) in Nrf2(-/-) Mutant Rats Fed High Salt Diet:** Because
low dose ANG II infusion reduces vascular oxidant stress and restores endothelium-
dependent vascular relaxation in HS-fed rats (33, 37, 38, 60, 64, 65) and hamsters (46),
we performed experiments to evaluate endothelial function in isolated middle cerebral
arteries (MCA) from wild type and Nrf2(-/-) mutant rats. In those experiments, MCA of
*Nrf2*(-/-) mutant rats and WT littermates maintained on low salt diet dilated to a similar
extent in response to ACh while HS diet eliminated ACh-induced dilation [as previously
reported in other experimental models (33, 37, 60)] in MCA from both WT and *Nrf2*(-/-)
mutant rats (Figure 5). Chronic infusion of ANG II restored ACh-induced dilation in MCA of WT controls fed short term HS diet, but this protective effect of ANG II infusion to restore endothelium-dependent vasodilation to ACh was completely absent in MCA of HS-fed Nrf2(-/-) mutant rats (Figure 6). MCA of Nrf2(-/-) mutant rats and WT littermates fed either LS or HS diet exhibited similar dilations in response to the NO-donor sodium nitroprusside (not shown), indicating that neither HS diet nor the Nrf2 mutation affect vascular smooth muscle NO sensitivity in this experimental model.

**Effect of Low Dose ANG II Infusion on Salt-Induced Microvascular Rarefaction in Nrf2(-/-) Rats:** Elevated dietary salt intake is also associated with a reduced density of microvessels (microvascular rarefaction) in Sprague-Dawley rats (18). Consistent with that report (18), microvessel density was significantly lower in the cremaster muscle of Sprague-Dawley (S-D) parental rats fed high salt diet for 3 days compared to LS controls; and microvascular rarefaction in S-D rats fed short term HS diet was prevented by subcutaneous infusion of a low dose of ANG II (100 ng/kg/min) (Figure 7). ANG II infusion also increased microvessel density in the cremaster muscle of wild-type rats fed short term HS diet, but not in cremaster muscles of HS-fed Nrf2(-/-) mutant rats (Figure 8).

**DISCUSSION**

In the present study, we developed a novel rat strain (SD-Nfe212mut1Mcwi) in which a deletion mutation of the Nrf2 gene was created in the Sprague-Dawley genetic background. We utilized this novel genetic rat strain to test the hypothesis that suppression of the NRF2 antioxidant defense system contributes to endothelial dysfunction arising from salt-induced ANG II suppression; and to evaluate the potential
role of NRF2 in contributing to the protective effect of low dose ANG II infusion to ameliorate endothelial dysfunction and prevent microvascular rarefaction in HS fed rats.

Although Nrf2 knockout mice exist, there is a substantially greater body of background knowledge regarding the physiology of the rat vs. the mouse, and the small size of the mouse presents substantial challenges for in vivo physiological studies because it precludes many of the experimental procedures that are extremely successful in the rat, e.g., blood pressure monitoring combined with serial blood sampling in conscious animals (15, 19, 34). In this regard, the availability of a Nrf2(−/−) mutant rat strain provides significant advantages for detailed physiological studies. Of particular importance in regard to the present experiments is that the renin-angiotensin system (RAS) in mice is very different from that in rats (4, 7), which more closely resembles that of the human (7). For example, ANG II infusion leads to a substantial increase in mean arterial pressure (MAP) in rats but not mice (4, 7) and aortic contractions in response to ANG II are substantially greater in rats vs. mice (4, 7). ACE inhibition and losartan produce a 50-90% greater fall in MAP in conscious mice vs. rats (7). ANG II causes medial hypertrophy in rat arteries but adventitial expansion in mouse arteries (4); and ANG II infusion reduces ANG II receptor density by 78% in mouse kidney and 29% in mouse spleen, with no effect in rats (4). In light of those findings, Cassis et al. (4) concluded that considerably higher doses of ANG II are necessary to elicit physiological effects in mice vs. rats. In light of existing reports that plasma ANG II levels in conscious mice and rats are not significantly different (7), the above findings strongly suggest that caution is necessary when utilizing mouse models to study the effects of physiological ANG II levels on the cardiovascular system.
In the present study, the deletion mutation in the first exon of the Nrf2 gene of the SD-Nfe212<sup>em1Mcwi</sup> rats included the start codon for NRF2 translation, and was transmitted to mRNA, as verified in cDNA by the presence of a truncated PCR product (Figure 1) and the absence of NRF2 protein evaluated by immunohistochemistry in whole kidney sections (Figure 2). Verification of this deletion mutation in the Nrf2(-/-) rat is important, as it supports the hypothesis that any changes in vascular reactivity or microvessel density in the Nrf2(-/-) mutant rats (or in the ability of low dose ANG II infusion to modify those parameters) compared to responses in wild type littermates are due to loss of the antioxidant and cell protective effects of the NRF2 protein in the mutant rats. Supporting the hypothesis that loss of NRF2 has an adverse effect on antioxidant mechanisms in mutant rats fed HS diet is the finding that the expression of mRNA for the NRF2-regulated indicator genes heme oxygenase-1 (Ho-1), catalase (Cat), superoxide dismutase 1 (Sod1), superoxide dismutase 2 (Sod2), and glutathione reductase (Gsr) was significantly lower in livers of HS-fed Nrf2(-/-) mutant rats compared to those of wild type controls (Figure 3).

Classically, high levels of ANG II exert pro-oxidant effects by stimulating the vascular NADPH oxidase isoforms and increasing the production of superoxide anions (14, 28, 39, 57). However, new evidence suggests that there is a paradoxical antioxidant role for plasma ANG II levels within the physiological range. For example, in salt-insensitive (for blood pressure) animal models, suppression of plasma ANG II by a HS diet leads to changes in vascular function similar to those occurring with high-ANG II models including oxidant stress (65, 66) and endothelial dysfunction (33), without changes in mean arterial blood pressure (10, 32, 38, 60). Infusion of a low (subpressor)
dose of ANG II to prevent salt-induced ANG II suppression (15, 64) not only restores endothelium-dependent vascular relaxation, but also reduces vascular oxidant stress in arteries of HS-fed rats (65) and hamsters (46). In the context of the present study, this is important because it demonstrates that exposure to chronically low levels of circulating ANG II (with HS diet or likely in low renin forms of hypertension) leads to vascular oxidant stress independent of the complicating effects of elevated blood pressure; and reinforces the importance of NRF2 in mediating the tonic effects of physiological ANG II levels in maintaining vascular antioxidant defenses under normal physiological conditions.

Surprisingly, the antioxidant effects of low dose ANG II infusion are mediated via the AT₁ receptor for ANG II (46). The protective effect of low dose ANG II infusion to restore endothelium-dependent vasodilation in salt-fed animals depends on transactivation of the epidermal growth factor receptor in response to ANG II binding to its AT₁ receptor, with subsequent activation of ERK 1/2 signaling and, ultimately, new protein synthesis (37). Consistent with this interpretation, studies in other laboratories have shown that ERK 1/2 signaling promotes the translocation of NRF2 to the cell nucleus in other experimental models (45, 67, 68). Taken together, these findings suggest that the protective effect of ANG II infusion to reduce vascular oxidant stress and restore endothelium-dependent dilation in salt-fed animals is mediated via activation of the NRF2 antioxidant defense system in response to AT₁ receptor activation and subsequent activation of the ERK 1/2 signaling pathway (37). In the present study, the observation that ANG II promoted translocation of NRF2 to the nucleus in cultured aortic endothelial cells from Sprague-Dawley parental rats (Figure...
is also consistent with the hypothesis that the NRF2 antioxidant defense system plays an important role in the protective effects of low dose ANG II infusion to restore endothelial function (33, 37, 46) (Figure 6) and prevent microvascular rarefaction (18) (Figure 8) in salt-fed animals.

Previous studies have shown that high salt diet leads to striking structural changes in arterioles and capillaries (16) and to a significant reduction in microvessel density (microvascular rarefaction) (18). Microvascular rarefaction in salt-fed animals is due to salt-induced ANG II suppression, and is prevented by restoring normal plasma ANG II levels via chronic infusion of a low dose of ANG II (18). In the present study, the failure of low dose ANG II infusion to restore microvessel density in the HS-fed Nrf2(-/-) mutant rats indicates that activation of NRF2-regulated antioxidant defenses plays a crucial role in mediating the protective effect of ANG II infusion to prevent microvascular rarefaction in HS-fed animals, and also suggests that tonic activation of the NRF2 antioxidant defense system may contribute to ability of physiological levels of ANG II to maintain normal microvessel density.

Yun and coworkers (62) postulated that angiogenic responses and collateral vessel formation are likely to depend upon an optimal level of reactive oxygen species (ROS) within a relatively narrow “redox window.” As such, the failure of low dose ANG II infusion to increase microvessel density in the cremaster muscle of Nrf2(-/-) rats could be due to elevated levels of ROS in the tissue as a result of reduced expression and activity of NRF2-regulated antioxidant enzymes in the tissue of the mutant rats. Supporting the latter hypothesis are the findings of Reed et al (50), who reported that low dose ANG II infusion improved collateral flow following repeated coronary occlusion
in WKY rats, but failed to improve collateral vessel formation in the JCR rat model of syndrome X characterized by elevated levels of tissue oxidant stress. By contrast, high dose ANG II infusion, expected to generate higher ROS levels in the tissue, abrogated coronary collateral formation in the WKY rats; and AT\textsubscript{1} receptor blockade with candesartan improved coronary collateral flow in the JCR rats, but failed to improve coronary flow in the WKY rats (50). In a similar fashion, low dose ANG II infusion in the presence of elevated levels of tissue oxidant stress would likely fail to restore increase microvessel density in the HS-fed \textit{Nrf2}\textsubscript{(-/-)} mutant rats.

In the vascular reactivity studies, endothelium-dependent dilation to acetylcholine was identical in MCA from \textit{Nrf2}\textsubscript{(-/-)} mutant rats and wild type littermates fed low salt diet (Figure 5), demonstrating that loss of NRF2 does not impair endothelial function in the absence of salt-induced ANG II suppression and oxidant stress. Consistent with previous reports in salt-fed Sprague-Dawley rats (32, 33, 37, 60) and hamsters (46), HS diet eliminated ACh-induced dilation of MCA from both the wild type and the \textit{Nrf2}\textsubscript{(-/-)} mutant rats. The most important and novel finding of the present study was that the protective effect of ANG II infusion to restore endothelium-dependent dilation to ACh that was present in MCA of HS-fed wild type controls was completely eliminated in the \textit{Nrf2}\textsubscript{(-/-)} mutant rats (Figure 6).

Overall, the results of the current study support the hypothesis that the protective effect of low dose ANG II infusion to restore vascular relaxation and prevent microvascular rarefaction in salt-fed rats is mediated via activation of the NRF2 antioxidant defense mechanism, and suggest a mechanism by which physiological ANG II levels play a protective role in the vasculature through the maintenance of redox
homeostasis. In this paradigm, binding of ANG II to the AT\textsubscript{1} receptor transactivates the epidermal growth factor receptor (37) leading to ERK 1/2-mediated phosphorylation of component(s) of the Nrf2/Keap1 complex and translocation of NRF2 to the cell nucleus where it binds to the antioxidant response element (ARE) consensus sequence in the promoter region of antioxidant and cell protective genes. NRF2 binding to the ARE would, in turn, ameliorate the oxidant stress occurring in response to HS diet (65) and restore redox homeostasis necessary for the preservation of normal endothelial cell function, notably endothelial [Ca\textsuperscript{2+}]\textsubscript{i} signaling (64) and NO release (31, 56, 64-66), and also maintain tissue ROS levels within the narrow “redox window” (62) necessary to preserve angiogenic responses, as postulated by Reed et al. (50) and Yun et al. (62).

One question that remains unanswered is the potential effects of ANG II-induced NRF2 activation on reactive oxygen species (ROS) production itself. Previous studies (65) have shown that inhibition of nitric oxide synthase, cyclooxygenase, and cytochrome P450 enzymes all lead to a reduction in vascular oxidant stress in small mesenteric resistance arteries of HS-fed S-D rats, suggesting that antioxidant defenses in HS fed rats are reduced and therefore unable to buffer ROS production by any enzyme that produces superoxide as part of its normal catalytic activity. However, elucidation of the effect of the NRF2 system in directly regulating ROS formation itself in resistance arteries (especially during salt-induced ANG II suppression) clearly appears to be an important area for future investigation.

The NRF2 oxidant stress response plays an important role in a variety of pathophysiological states including diseases of the lung (5, 48, 49) and liver (44, 61), neurodegeneration (59), and cancer (43, 47, 51, 52). In humans, Nrf2 mRNA and
protein are down-regulated in alveolar macrophages collected from patients with chronic obstructive pulmonary disease (COPD) and long-time smokers (35, 55). Recently Marczak and coworkers (36) reported that the -653G polymorphism within the NRF2 promoter region is associated with reduced forearm blood flow and increased vascular resistance in healthy African-American volunteers; and also significantly reduces NRF2 transcription \textit{in vitro} (36).

Although oxidant stress is a hallmark of multiple cardiovascular diseases, clinical trials involving direct administration of antioxidants have been surprisingly disappointing (42). As a result, there is growing interest in direct upregulation of endogenous antioxidant systems, including NRF2, as a potential therapeutic approach to pathological conditions characterized by increased oxidant stress. For example, in whole animal models, inducers of NRF2 expression attenuate ischemic damage from stroke and mitochondrial stress (1, 53, 54). Sulforaphane-induced NRF2 activation also improves many aspects of blood-brain barrier function following brain injury produced by controlled cortical impact, including maintenance of endothelial cell markers, maintenance of tight junction proteins in the microvasculature, and decreased edema—indicative of a less permeable blood-brain barrier \textit{in vivo} (63).

By employing a novel \textit{Nrf2} mutant rat strain, we have shown that NRF2 is necessary for the protective effect of low dose ANG II infusion to ameliorate endothelial dysfunction and prevent microvascular rarefaction in the face of an elevated dietary salt intake. However, the potential importance of the \textit{Nrf2} \textit{(-/-)} mutant rat strain extends far beyond insight into the novel protective role of physiological levels of ANG II in maintaining normal endothelial function because of the global nature of the NRF2
system. For example, Hybertson et al. (21) noted that dysregulation of NRF2-regulated
genes provides a logical explanation for the direct and indirect connections between
oxidant stress and more than 200 human diseases; and that NRF2 can modulate the
expression of hundreds of different genes that affect multiple processes ranging from
immune and inflammatory responses, tissue remodeling and fibrosis, carcinogenesis
and metastasis, and even cognitive dysfunction. As such, direct upregulation of the
NRF2 antioxidant defense system could prove to be a highly beneficial therapeutic and
preventative strategy for pathophysiological conditions associated with increased
oxidant stress; especially in light of the disappointing results of clinical trials involving
direct administration of antioxidants (42). In this regard, crucial insights into the role of
NRF2 in these processes and the mechanisms of NRF2 action could be obtained from
studies of the SD-Nfe212^{em1Mowi} mutant rat strain, or other Nrf2^{(-/-)} mutant rat strains
developed in disease-sensitized genetic backgrounds.
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6. Chan K, Lu R, Chang JC, and Kan YW. N RF 2 , a member of the NFE2 family of transcription factors, is not essential for murine erythropoiesis, growth, and development. Proc Natl Acad Sci USA 93: 13943-13948, 1996.


FIGURE LEGENDS

**Figure 1.** Effect of Nrf2 gene mutation on cDNA from liver and kidney. An expected product of 389 bp is represented in wild type (WT) tissues. TALEN-mediated mutagenesis resulted in a 41 base deletion, represented by the smaller 348 bp band in homozygous mutant tissues. Organs were from HS-fed, saline-infused rats.

**Figure 2.** Effect of Nrf2 gene mutation on immunohistological detection of NRF2 in the whole kidney. NRF2 staining (brown) is markedly absent from renal structures (A, for artery, G, for glomerulus, and RT for renal tubule) in the kidneys of low salt-fed Nrf2(-/-) mutant rats (right) compared to low salt-fed wild type (WT) animals (left). Upper panels were photographed with a 20X objective and bottom images are enlargements of the areas indicated by the white boxes in the top images. Scale bar in upper panel = 200 µm.

**Figure 3.** Expression of mRNA for the NRF2-regulated indicator enzymes catalase (Cat), heme oxygenase-1 (Ho-1), superoxide dismutase 1 (Sod1), superoxide dismutase 2 (Sod2), and glutathione reductase (Gsr) in livers of Nrf2(-/-) mutant rats fed HS diet for 2 weeks compared to those from HS-fed wild type (WT) controls. Data are expressed as mean % of wild type control ± SEM for 3 animals. *--P<0.05—Student t-test.
**Figure 4.** NRF2 staining (green) and nuclear DAPI staining (blue) in aortic endothelial cells cultured from Sprague-Dawley rats fed high salt (HS; 4% NaCl) diet incubated with vehicle (left panel) or 100 nM ANG II for 24 hours (right panel).

**Figure 5.** Responses to the endothelium-dependent vasodilator acetylcholine in middle cerebral arteries (MCA) of Nrf2(-/-) mutant rats (KO) and wild type littermates (WT) maintained on a low salt (LS; 0.4% NaCl) diet or short-term (3 days) high salt (HS; 4% NaCl) diet. Data are summarized as mean percent of maximum dilation in Ca2+-free PSS (±SEM) for 4-5 animals per group. *-P<0.05 WT LS diet vs. WT HS diet; †-P<0.05 KO LS diet vs. KO HS diet—2 way ANOVA with Student Newman-Keuls test post hoc.

**Figure 6.** Responses to endothelium-dependent vasodilator acetylcholine in middle cerebral arteries (MCA) of Nrf2(-/-) mutant (KO) rats and wild type littermates (WT) fed high salt (HS; 4% NaCl) diet and infused with either saline vehicle or low-dose (100 ng/kg/min) ANG II via subcutaneous osmotic minipump for three days. Data are summarized as mean percent of maximum dilation in Ca2+ free PSS (±SEM) for 4-5 animals per treatment group. *-P<0.05 WT HS diet + ANG II vs. KO HS diet + ANG II; †-P<0.05 WT HS diet + ANG II vs. WT HS diet + saline—2 way ANOVA with Student Newman-Keuls test post hoc.

**Figure 7.** Effect of HS diet on microvessel density in cremaster muscles of Sprague-Dawley (S-D) parental rats fed low salt (LS; 0.4% NaCl) diet and high salt (HS; 4% NaCl) diet...
NaCl) diet for 3 days (± ANG II infusion—100 ng/kg/min, s.c.). Data are expressed as mean % of low salt control ± SEM for 5-6 animals. *-P<0.05 vs. low salt control; † P<0.05 vs. HS; ‡ P<0.05 vs. HS + isotonic saline vehicle—one way ANOVA with Kruskal-Wallis test post hoc.

**Figure 8.** Effect of low dose ANG II infusion (100 ng/kg/min, s.c.) on microvessel density in the cremaster muscle of wild-type (WT) and Nrf2(-/-) mutant (KO) rats fed high salt (HS; 4% NaCl) diet for 3 days. Scale bar=110 µm. Data are expressed as mean percent of wild type-saline infused control. *--P<0.05 vs. HS-fed controls infused with isotonic saline vehicle—one way ANOVA with Kruskal-Wallis test post hoc.
Table 1. Baseline Parameters in Wild Type and \textit{Nrf2}\textsuperscript{(-/-)} Mutant Rats*

<table>
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<th>Low Salt Diets</th>
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<th>High Salt Diets</th>
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<tr>
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<td>ANG II-Infused</td>
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<td>\textit{Nrf2} Mutant</td>
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*--No significant differences between groups for any of the variables-P>0.05 one way analysis of variance.
* Significantly different from Nrf2WT $P < 0.05$
Acetylcholine Concentration (Log mol/L)

Percent Maximum Dilation (%)

WT LS Diet (n=4)
KO LS Diet (n=4)
WT HS Diet (n=5)
KO HS Diet (n=4)

* p<0.05 for WT LS Diet vs. WT HS Diet
† p<0.05 for KO LS Diet vs. KO HS Diet
Acetylcholine Concentration (Log mol/L)

Percent Maximum Dilation (%)

-4 -2 0 2 4 6 8 10

NRF2 WT HS Saline (n=5)
NRF2 WT HS ANG-II (n=4)
NRF2 KO HS Saline (n=4)
NRF2 KO HS ANG-II (n=5)

* p<0.05 for NRF2 WT HS ANG-II vs. NRF2 KO HS ANG-II
† p<0.05 for NRF2 WT HS Saline vs. NRF2 WT HS ANG-II
Microvessel Density (%LS Control)

<table>
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<tr>
<th>Treatment</th>
<th>LS</th>
<th>HS</th>
<th>HS+Saline</th>
<th>HS+AngII</th>
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<tr>
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<td>LS</td>
<td>HS</td>
<td>HS+Saline</td>
<td>HS+AngII</td>
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</table>

* p<0.05 compared to LS
† p<0.05 compared to HS
‡ p<0.05 compared to HS + Saline
Microvessel Density (% of WT HS Saline)

WT HS Saline (n=5)  WT HS ANG-II (n=5)  KO HS Saline (n=5)  KO HS ANG-II (n=5)

* p<0.05 vs WT HS Saline, KO HS ANG-II  Strain/Infusion