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Mutation within the hinge region of the transcription factor *Nr2f2* attenuates salt-sensitive hypertension

Sivarajan Kumarasamy^{1,2}, Harshal Waghulde^{1,2}, Kathirvel Gopalakrishnan^{1,2}, Blair Mell^{1,2}, Eric Morgan^{1,2,3}, and Bina Joe^{1,2}

¹Center for Hypertension and Personalized Medicine, University of Toledo College of Medicine and Life Sciences, Toledo, Ohio, USA

²Department of Physiology and Pharmacology, University of Toledo College of Medicine and Life Sciences, Toledo, Ohio, USA

³Center for Diabetes and Endocrine Research, University of Toledo College of Medicine and Life Sciences, Toledo, Ohio, USA

Abstract

Genome-wide association studies (GWAS) have prioritized a transcription factor, Nuclear Receptor 2 Family 2 (*NR2F2*), as being associated with essential hypertension in humans. Here we provide evidence that validates this association and indicates that *Nr2f2* is a genetic determinant of blood pressure (BP). Using the zinc-finger nuclease technology, the generation of a targeted *Nr2f2*-edited rat model is reported. The resulting gene-edited rats have a 15bp deletion in exon 2 leading to a 5 amino acid deletion in the hinge region of the mutant *Nr2f2* protein. Both systolic and diastolic blood pressures of the *Nr2f2^{mutant}* rats are significantly lower than controls. Because the hinge region of *Nr2f2* is required for interaction with Friend of Gata2 (*Fog2*), protein-protein interaction is examined. Interaction of *Nr2f2^{mutant}* protein with *Fog2* is greater than that with the wild type *Nr2f2* indicating that the extent of interaction between these two transcription factors critically influences BP.

Introduction

BP is a complex polygenic trait influenced by multiple genetic elements, the number, magnitude of effect and identities of which are largely unknown. Genome-wide association studies have prioritized genes, based on their association with hypertension, as potential candidates for BP regulation. The Wellcome Trust Case-Control Consortium (WTCCC) was

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To whom correspondence should be addressed: Bina Joe, Ph.D., Program in Physiological Genomics, Center for Hypertension and Personalized Medicine, Department of Physiology and Pharmacology, University of Toledo College of Medicine and Life Sciences, 3000 Arlington Avenue, Toledo, Ohio 43614-2598, USA; Phone (419) 383-4144, Fax (419) 383-2871, bina.joe@utoledo.edu.

Author Contributions

B.J. conceived and designed the project. S.K., H.W., K.G., E. M., and B.M. performed the experiments. S.K., E.M., and H.W., analyzed the data. B.J., S.K., K.G., E.M., and H.W., analyzed the data. B.J., S.K., E.M., and H.W. prepared the manuscript.

Competing Financial Interest

The authors declare no competing financial interest.

one of the first large scale GWAS to report a suggestive association on a region on human chromosome 15 to hypertension¹. Subsequently, by a haplotype-based re-analysis of this data the gene nuclear receptor subfamily 2, group F, member 2 (*NR2F2*) also known as Chicken ovalbumin upstream promoter transcription factor (*COUP-TFII*) was highly prioritized as a candidate gene associated with hypertension². Four other linkage studies in humans have reported the genomic region containing *NR2F2* as being linked to essential hypertension³⁻⁶. Similar lines of evidence were obtained with multiple linkage and substitution mapping studies using rat models of hypertension⁷⁻¹¹. *Nr2f2* is a nuclear receptor transcription factor vital for angiogenesis and heart development¹², but its role in BP regulation remains undefined.

The current study was conducted to directly test the involvement of *Nr2f2* in BP regulation. For this purpose, a targeted gene-edited rat model was generated, in which the *Nr2f2* protein was mutated in the hinge region. Both systolic and diastolic blood pressures (BP) of the *Nr2f2^{mutant}* rats are significantly lower than controls, indicating that *Nr2f2* is indeed a genetic determinant of BP. Further, the structural mechanism by which *Nr2f2* influences BP is delineated to the hinge region, through which it interacts with another transcription factor, Friend of Gata2 (*Fog2*). The extent of interaction of *Nr2f2* with *Fog2* is enhanced when the *Nr2f2* protein is mutated in the hinge region. These data provide evidence for the extent of interaction between two transcription factors, *Nr2f2* and *Fog2* as being important for the regulation of blood pressure.

Results and Discussion

Targeted-editing of the *Nr2f2* locus

Gene deletion mouse models serve as genetic tools to validate observations documented through genetic linkage and association studies. *Nr2f2* knockout mice are, however, embryonic lethal¹³. To examine the role of *Nr2f2* in BP regulation, we therefore focused on constructing a *Nr2f2^{mutant}* rat model without disrupting the two important domains of the *Nr2f2* protein, the DNA-binding domain and the ligand-binding domain. Custom- zinc-finger nucleases (ZFNs) targeting exon 2 of *Nr2f2* were designed. Exon 2 of *Nr2f2* codes for amino acids in the hinge region of the *Nr2f2* protein, which is between the DNA-binding and the ligand-binding domains. ZFNs targeting *Nr2f2* were microinjected into single-cell embryos of the hypertensive rat strain- the Dahl Salt-sensitive (S) rat. Embryonic lethality was bypassed by the hypomorphic founder *Nr2f2^{mutant}* rats. These rats had a shorter (345 bp) PCR-amplified genomic DNA fragment encompassing exon 2 of *Nr2f2* compared with the wild-type *Nr2f2* (360 bp) genomic fragment (Figure 1A). Sequencing of the PCR products confirmed the deletion to be 15bp from 1571 to 1556 bp of the mRNA of *Nr2f2* (Fig. 1B). Two different antibodies against *Nr2f2* were used to clarify the protein status of *Nr2f2*. Immunoblotting with an N-terminal antibody detected the presence of *Nr2f2* protein in both the *Nr2f2^{mutant}* rats and the wild type hypertensive rats, whereas, antibodies to the hinge region of *Nr2f2* cross-reacted exclusively with the wild-type *Nr2f2* protein but not the mutant *Nr2f2* protein (Fig. 2, Supplementary Figure 1).

Attenuation of hypertension in the *Nr2f2*^{mutant} rats

Compared to the wild-type hypertensive rats, *Nr2f2*^{mutant} rats maintained on a high salt (2% NaCl) diet had lower systolic BP (179±3 vs. 197±5 mmHg; t-test, p<0.05) as measured by the tail-cuff method. To further confirm these observations, rats were surgically implanted with radio transmitters and BP was continuously recorded by telemetry. Both the *Nr2f2*^{mutant} rats and the wild type hypertensive rats maintained normal diurnal rhythms of systolic and diastolic BP. However, throughout the observation period, both systolic and diastolic BP of the *Nr2f2*^{mutant} rats were consistently lower than that of the wild type hypertensive rats (Figs. 3A, B). *Nr2f2*^{mutant} rats maintained on a low salt (0.3% NaCl) diet did not however demonstrate a similar BP lowering effect (Supplementary Fig. 2). Figures 3C and 3D demonstrate the blunted increase in salt-induced blood pressure in the mutant rats compared to the hypertensive rats. These data constitute strong evidence to indicate that the structural alterations within the hinge-region of *Nr2f2* contribute importantly to salt-sensitive BP regulation.

Improved cardiac and renal function of the *Nr2f2*^{mutant} rats

Nr2f2^{mutant} rats exhibited superior cardiac function compared with the wild type hypertensive rats. As measured by echocardiography, *Nr2f2*^{mutant} exhibited superior left ventricular function as demonstrated by increased fractional shortening (FS) (56%+/-3 vs. 41%+/-2; t-test, p<0.05, Fig. 4A), increased velocity of circumferential shortening (Vcf) (8.6+/-0.3 vs. 6.1+/-0.4; t-test, p<0.05, Fig. 4B) and increased FS/MPI (Fractional shortening/Myocardial performance index) (1.48+/-0.22 vs. 0.78+/-0.08; t-test, p<0.05, Fig. 4C). *Nr2f2*^{mutant} rats also exhibited superior renal function compared with the wild type hypertensive rats. Urinary protein excretion of *Nr2f2*^{mutant} rats was lower compared with the wild type hypertensive rats (87.97±7.07 vs. 122.63±7.07; t-test, p<0.05) (Fig. 5). These additional physiological parameters that provide evidence to the overall improvement in cardiovascular and renal function that are associated with the observed lowering of BP in the *Nr2f2*^{mutant} rats.

Interaction between *Nr2f2* and *Fog2* is linked to BP regulation

Next, we sought to explore the molecular mechanism by which *Nr2f2* mediates the observed alterations in blood pressure, we focused on the sole difference between the two strains, which is in the hinge region of *Nr2f2*. Amino acids 117–414 of *Nr2f2* are required for protein-protein interaction with another transcriptional regulator, Friend of Gata2 (*Fog2*)¹⁴. *Fog2* is a multi-zinc finger transcriptional co-repressor protein required for cardiac development. *Fog2* regulates cardiac morphogenesis by interaction with multiple cardiac specific transcriptional factors. *Nr2f2* and *Fog2* null mice have similar defects related to mesenchymal-epithelial and endothelial interaction, which are important for heart and blood vessel formation¹⁴. Of further interest, Ciullo *et al.*, identified a new locus strongly linked to hypertension in human chromosome 8q22-23 region, within which, *Fog2* is prioritized as one of the candidate genes for essential hypertension¹⁵. We therefore hypothesized that the protein-protein interaction between the hinge region of *Nr2f2* and *Fog2* is important for BP regulation. To test this hypothesis, wild type and mutant forms of *Nr2f2* cDNA, with Histidine tags, were cloned into pCDNA 3.1(B) myc/His (-) expression vector and

overexpressed in NRK52E or COS-7 cells (Fig. 6A, Supplementary Fig. 3). The overexpressed Nr2f2 proteins along with their binding partners were immunoprecipitated with the Nr2f2 N-terminal antibody or with histidine tags and probed with the antibody against Fog2. Compared with the wild-type Nr2f2 protein, the binding of the mutant form of Nr2f2 to Fog 2 was more pronounced (Figure 6B). To further assess the downstream effect of this interaction, we conducted a promoter chromatin immuno-precipitation experiment. As shown in Figure 7, an enrichment of Nr2f2 binding to the promoter of atrial natriuretic factor, *Anf*, was noted in the Nr2f2^{mutant} rats. *Anf* is a direct target gene known to be influenced by the Nr2f2-Fog2 interaction¹⁴. Similar enrichments were not observed with promoters for *renin* and *ApoB*, which are other targets of Nr2f2^{16,17} that are not described in the literature to require Fog2. Because *Anf* is a vasorelaxant^{18,19}, rats were further tested for vasoreactivity. Vasorelaxation responses of the mesenteric arteries from Nr2f2^{mutant} rats were significantly higher than that of the wild-type rats (Fig. 8). These data indicate that the extent of interaction between Nr2f2 and Fog2 through the hinge region of Nr2f2 is linked to the extent of blood pressure and that modulation of transcription of *Anf*, is perhaps, one of the potential underlying mechanisms.

In summary, our study provides an important step forward in our current understanding of *Nr2f2* as a genetic factor associated with essential hypertension in humans and in rodent models of hypertension. Specifically, combined with the available linkage studies in rat models, by utilizing a novel mutant model of *Nr2f2*, we have not only demonstrated that *Nr2f2* is a genetic determinant of BP, but also documented the significance of the relatively less-explored hinge region of Nr2f2 as an important molecular feature, which through its interaction with Fog2, is linked to BP regulation.

METHODS

Animals

All animal procedures and protocols used in this report were approved by the University of Toledo Health Science Campus Institutional Animal Care and Use Committee.

Generation of a ZFN-Mediated *Nr2f2*^{mutant} Rat

Zinc- finger nuclease (ZFN) construct specific for the rat *Nr2f2* gene (NM_080778.2) were designed, assembled, and validated by Sigma-Aldrich, to target the first exon 2 of *Nr2f2* (Target sequence: CAGGATGCCGCCACCCAgcctacTCACGGGCAGTTTGCACTG; ZFN binds to each underlined sequence on opposite strands). mRNA encoding the *Nr2f2* ZFN pairs at a concentration of 10 ng/uL were injected into one cell SS/McwiHsd (SS) rat embryos²⁰. Out of a 147 embryos injected, 66 healthy embryos were transferred to pseudo-pregnant female SD:CrI rats (Charles River), of which five pups were born. At 14 days of age, pups were tagged, tail clipped and DNA was extracted and amplified using primers flanking the above target sequence *Nr2f2*-F (5' GGGGAAGTGATTTGTGAGGA 3') and *Nr2f2*-R (5' GCCCATGATGTTGTTAGGCT 3'). PCR products were analyzed for ZFN-induced mutations using a Cel-I assay as described previously²⁰. Among the 5 pups born, 2 positive founders (male/female) were identified. PCR products were TOPO cloned and sequenced to reveal the same 15-bp deletion 'CCTACTCACGGGCAG' in exon 2 of the

Nr2f2 gene in both mutant animals. The founder male/female rats were backcrossed to the parental SS/JrHsdMcwi strain. Multiple separate pairs of mutation-carrying progeny were then intercrossed to generate an F2 population that was used for phenotyping and breeding to homozygosity. The DNA sequencing of the homozygous *Nr2f2^{mutant}* rats confirmed the 15-bp deletion in F2 offspring, which results in a predicted loss of five amino acids PTHGQ.

Blood Pressure Measurements by the tail-cuff and radiotelemetry methods

Each set of wild-type hypertensive SS/JrHsd Mcwi rats, hitherto referred as hypertensive rats ($n = 20$ males) and *Nr2f2^{mutant}* rats ($n = 17$ males), hypertensive rats ($n = 6$ females) and *Nr2f2^{mutant}* rats ($n = 6$ females) were bred, housed, and studied concomitantly to minimize environmental effects. All rats were fed with 0.3% NaCl low-salt diet (Harlan Teklad), weaned at 30 days of age. To assess salt-sensitivity, at the age of six weeks rats were divided into two groups. The low salt group was continued to be fed with the 0.3% NaCl low-salt diet, whereas the high salt group was switched to a diet containing 2% NaCl. After 24 days on these diets, systolic blood pressure (BP) of the rats was measured by using the tail-cuff method²¹. Conscious restrained rats were warmed to 28°C. The BP of each rat was measured for 2 consecutive days by two blinded operators. BP values for each day were the mean of three or four consistent readings. The final BP value used was the mean of the two daily BP values. BP was also collected using a telemetry system (Data Sciences International, St. Paul, MN)²². Four days after the BP measurements by the tail cuff method, *Nr2f2^{mutant}* ($n = 8$ males) and wild-type hypertensive rats ($n = 7$ males) rats were surgically implanted with C40 radio-telemetry transmitters such that the body of the transmitter is placed into the left flanks and the probe is inserted into their femoral arteries all the way into the lower abdominal aortae. Rats were allowed to recover from surgery for 4 days before the transmitters were turned on for recording BP.

Urinary Protein Excretion (UPE)

Within 5 days following BP measurements, *Nr2f2^{mutant}* ($n=17$) and wild-type hypertensive ($n=18$) rats were housed individually in metabolic cages and urine samples were collected over a 24 hr period. The pyrogallol based QuanTtest Red Total Protein Assay from Quantimetrix (Redondo Beach, CA) was used to determine protein concentrations of the urine samples. A VERSAmax microplate reader from Molecular Devices (Sunnyvale, CA) was used to determine absorbance at 600nm. Protein concentrations were determined by reading against the absorbance of the QuanTtest human protein standards (25–200 mg/dL). UPE data is presented as milligrams of protein/kg of body weight over a 24h period (mg of protein/kg bwt/24h).

Echocardiography

Nr2f2^{mutant} ($n = 12$) and wild-type hypertensive rats ($n = 12$) were evaluated by transthoracic echocardiography for determination of fractional shortening (FS), velocity of circumferential shortening (Vcf), and myocardial performance index(MPI) using a Sequoia C512 System (Siemens Medical) with a 15-MHz linear array transducer^{23,24}. The rats were anesthetized with 1.5–2.0% isoflurane by mask, the chest was shaved, the animal was situated in the supine position on a warming pad and ECG limb electrodes were placed.

Two-dimensional guided M-mode images and Doppler studies of aortic and trans-mitral flows were obtained from the parasternal long and foreshortened apical windows, respectively. Study duration was typically 15–20 min per animal.

All data were analyzed offline with software resident on the ultrasound system in an investigator-blinded fashion. LV end-diastolic (LVDd) and end-systolic (LVSD) dimensions were determined from M-mode tracings recorded at the mid-papillary level, and LV fractional shortening (FS) was calculated as $[(LVDd-LVSD)/LVDd] \times 100\%$. Ejection time and the isovolumic contraction and relaxation times were measured from color-flow directed Doppler pulsed-wave traces of mitral and aortic flow obtained at the level of the LV outflow tract from the apical four-chamber view. Aortic outflow and mitral inflow waveforms were recorded when the mitral and aortic wave forms were distinct and aortic and mitral valve clicks were clearly visible. Ejection, contraction, and relaxation times were determined from three consecutive beats and averaged to calculate MPI. MPI was calculated as the sum of the isovolumic contraction and relaxation times divided by the ejection time. The velocity of circumferential shortening (Vcf) was calculated as FS/ejection time. Values are means \pm SE. Groups were compared with Student's *t*-test.

Vascular Reactivity

After recording blood pressure by radiotelemetry the wild-type hypertensive (n=9) and *Nr2f2^{mutant}* rats (n=4) were euthanized with CO₂ inhalation method. The mesentery was immediately excised and placed in ice-cold physiological salt solution (PSS) consisting of mmol/L of 118.5 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 MgSO₄, 1.2 KH₂PO₄, 25.0 NaHCO₃, and 5.5 D-glucose aerated with 95% O₂-5% CO₂. Using a dissecting microscope, the mesenteric arteries were gently cleaned of adipose and connective tissue and cut into segments of approximately 2 mm in length. The segments were then mounted in a Mulvany-Halpern style small vessel wire myograph chamber (Model 620M, Danish Myotechnology, Aarhus, Denmark) filled with 6 ml PSS, maintained at 37°C, and continuously aerated with 95% O₂, 5% CO₂ to measure vascular reactivity^{25–27}. After 30 min of equilibration period, a standardized computer-assisted normalization procedure was performed to set the pretension of the arteries using LabChart8 software (AD Instruments, Colorado Springs, CO). This involves setting a circumference of the arteries equivalent to 90% of that which they would have at a transmural pressure of 100mmHg. Segments were washed with PSS and left to equilibrate for another 30 min. Vessel contractility was then assessed with the high-K⁺ (120mmol/L) solution. After washing the segment 3–4 times with PSS, cumulative concentration-response curve (CCRC) to phenylephrine (0.1–100μM) was obtained. After another 30-min wash, the segments were precontracted with submaximal dose of phenylephrine until the plateau was achieved. Dose-dependent relaxation responses to ACh (0.001–1μM) and sodium nitroprusside (SNP) (1nM–100μM) were assessed by constructing CCRC. Relaxation in response to ACh and SNP were expressed as a percentage of the level of pre-constriction induced by submaximal dose of phenylephrine.

Western blotting (WB)

Antibodies were from the following sources: Anti-Nr2f2 mouse monoclonal (Nr2f2-Ab1, Abcam, 1:2000 dilution), anti-Couptf II (Nr2f2-Ab2, Cell signaling, 1:500 dilution), anti-

Fog2 (GeneTex, 1:1000 dilution), donkey anti-mouse IgG-HRP conjugate (SC-2020, 1:5000 dilution), anti-rabbit IgG-HRP conjugate (Cell Signaling, 1: 5000 dilution). Samples were homogenized in ice-cold RIPA lysis buffer with protease inhibitor cocktail (Pierce). Immuno-blotting for Nr2f2 was carried out using tissue lysates from *Nr2f2^{mutant}* and wild-type hypertensive rat homogenates. In brief, forty microgram of proteins were boiled with Laemmli sample buffer (Bio-Rad) for 5 minutes at 95° C. Protein samples were resolved using 4–15% Criterion Tris-HCl Gel (Bio-Rad) at room temperature, and transferred on to PVDF membrane (Millipore), blocked with 5% fat free milk powder in PBST and incubated with anti-Nr2f2 and secondary antibodies conjugated with horseradish peroxidase. Immunoreactivity was detected by autoradiography using SuperSignal West Pico Chemiluminescent Substrate (Pierce). Full-length blots are shown in Supplementary Figure 1.

Chromatin Immunoprecipitation (ChIP)

ChIP^{16,28} was performed using heart tissue from wild-type male hypertensive (n=3) and *Nr2f2^{mutant}* rats (n=3). Tissues was minced and fixed with 1% formaldehyde. After crosslinking, nuclei were isolated and the cross-linked chromatin was subjected to sonication to obtain DNA fragments. Chromatin (50µg) was immunoprecipitated with 8µg of Nr2f2 antibody (Nr2f2-Ab1). As a negative control, chromatin was precipitated with 8µg of mouse IgG (Cell signalling). The immune complexes were precipitated with protein G-Agarose beads. Precipitated chromatin was eluted from the beads and crosslinks were reversed overnight at 65°C. Chromatin was treated with RNase A, proteinase K, and the DNA was column purified (PCR Purification kit, Qiagen). Purified DNA was PCR amplified using primers (Supplementary Table 1) targeting the *Anf*, *Renin* and *Apob* promoter regions, and *Hbb* used as a negative control.

Cell culture, transfection and immunoprecipitation

NRK52E cells were obtained from ATCC (Catalog number CRL-1571) and cultured in DMEM supplemented with 5% fetal bovine serum, 100units/ml penicillin and 100µg/ml streptomycin in a humidified atmosphere at 37 °C with 5% CO₂. Both wild type and mutant forms of *Nr2f2* cDNA were cloned into the pCDNA 3.1(B) myc/His (-) expression plasmid. NRK52E cells were transfected with both wild-type and mutant forms of Nr2f2 plasmids using X-tremeGENE HP DNA transfection reagent (Roche Applied Science). Forty eight hours after transient transfection, cells were treated with the cell lysis buffer and used for subsequent experiments. For immunoprecipitation, lysates (500µg of protein) were pre-cleared with protein G agarose beads (Pierce) at 4°C for 2h. After removal of the beads by centrifugation, lysates were incubated overnight with anti-Nr2f2 antibody (5µg/IP experiment, Nr2f2-Ab1, Abcam) at 4°C. The immunoprecipitated complex was captured by incubating with 50 µl of protein G agarose beads. Western blotting was performed with Anti-Fog2 antibody (1:1000 dilution) as detailed above.

Statistical Analysis

All BP statistical analyses were done using the SPSS software (SPSS, Chicago, IL). Data were analyzed by independent sample t-test. Data are presented as the mean ± standard

error. A p -value of 0.05 was used as a threshold for statistical significance. For BP and UPE experiments measurements the number of rats in each group ranged from 6 to 20.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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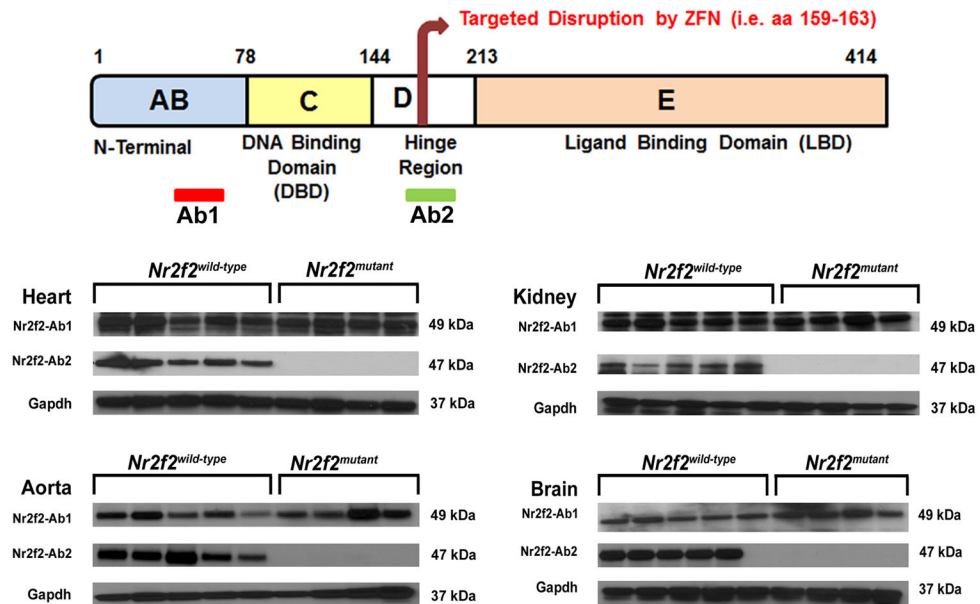


Figure 2. Targeted disruption of *Nr2f2*

Top: Schematic diagram of *Nr2f2* protein, highlighting the key domains: AB domain (aa 1-78); C domain or DNA binding domain (aa 79-144); D domain or Hinge region (aa 145-213); E domain or Ligand binding Domain (aa 214-414). Bottom: Western blot (WB) analysis of *Nr2f2* protein expression in *Nr2f2*^{mutant} and Wild-type hypertensive rats. Anti-*Nr2f2* antibodies are labeled as Ab1 and Ab2. Ab1: Antibody from Abcam, raised against a synthetic peptide directed towards amino acids 43–64 (Abcam); Ab2: Antibody (Cell Signaling) raised against a synthetic peptide directed towards the hinge region (amino acids around 159).

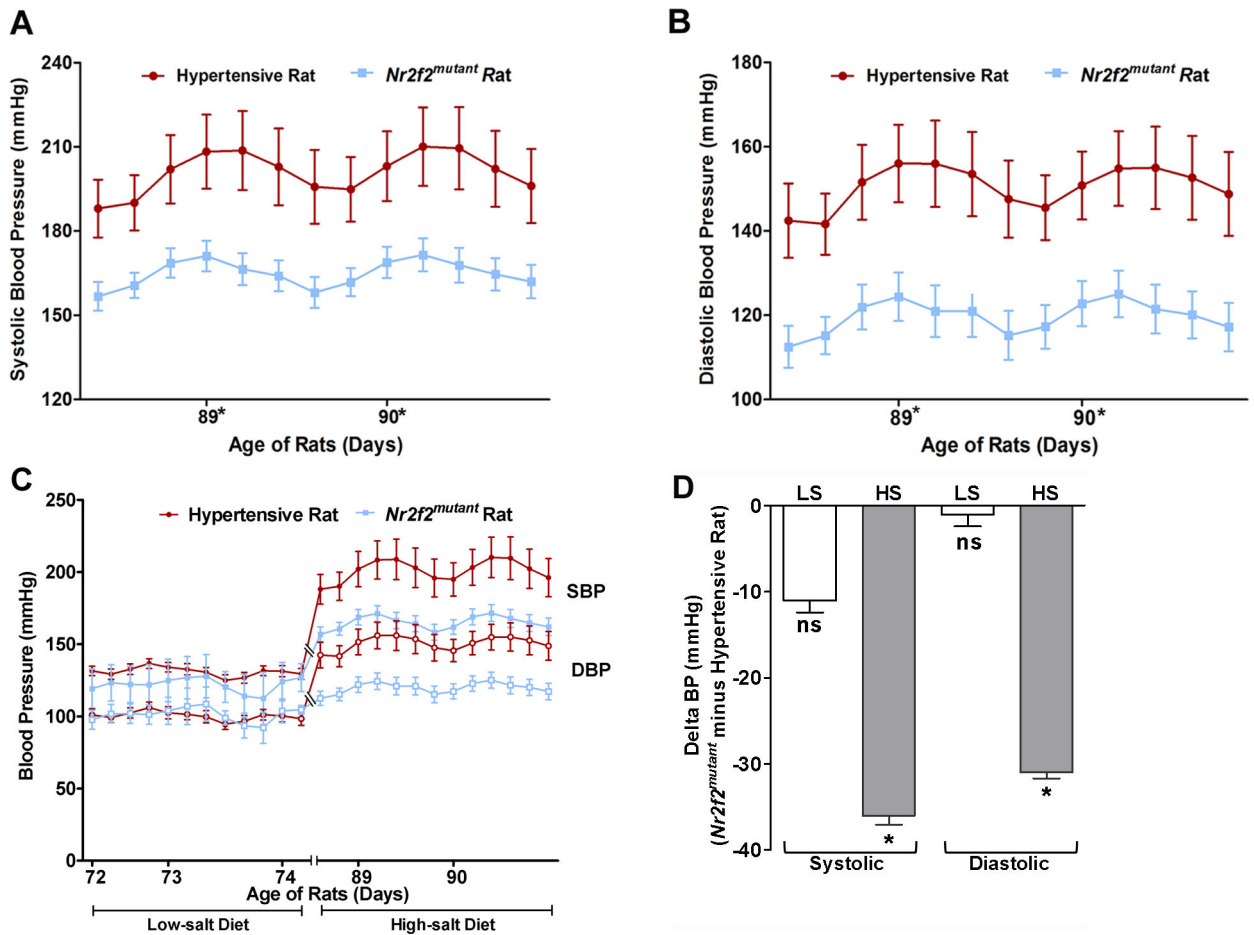


Figure 3. Lower Blood Pressure of *Nr2f2*^{mutant} rats

Measurements of (A) Systolic and (B) Diastolic blood pressures of hypertensive rats ($n = 7$) and *Nr2f2*^{mutant} rats ($n = 8$). Rats were monitored for BP after surgical implantation of radiotelemetry transmitters. Data plotted are the recordings obtained once every 5 min continuously for 24 h and averaged for 4 h intervals; (C) Comparison of low-salt versus high-salt diet regimens. Data presented from low-salt and high-salt fed rats was obtained from two independent experiments. (D) Delta BP calculated by subtracting the average BP of the hypertensive rat from the average BP of *Nr2f2*^{mutant} rats within each of the dietary salt groups labeled as LS for low-salt diet and HS for high-salt diet. Levels of statistical significance for all data were analysed by independent sample t-test. * $P < 0.05$, NS Not significant.

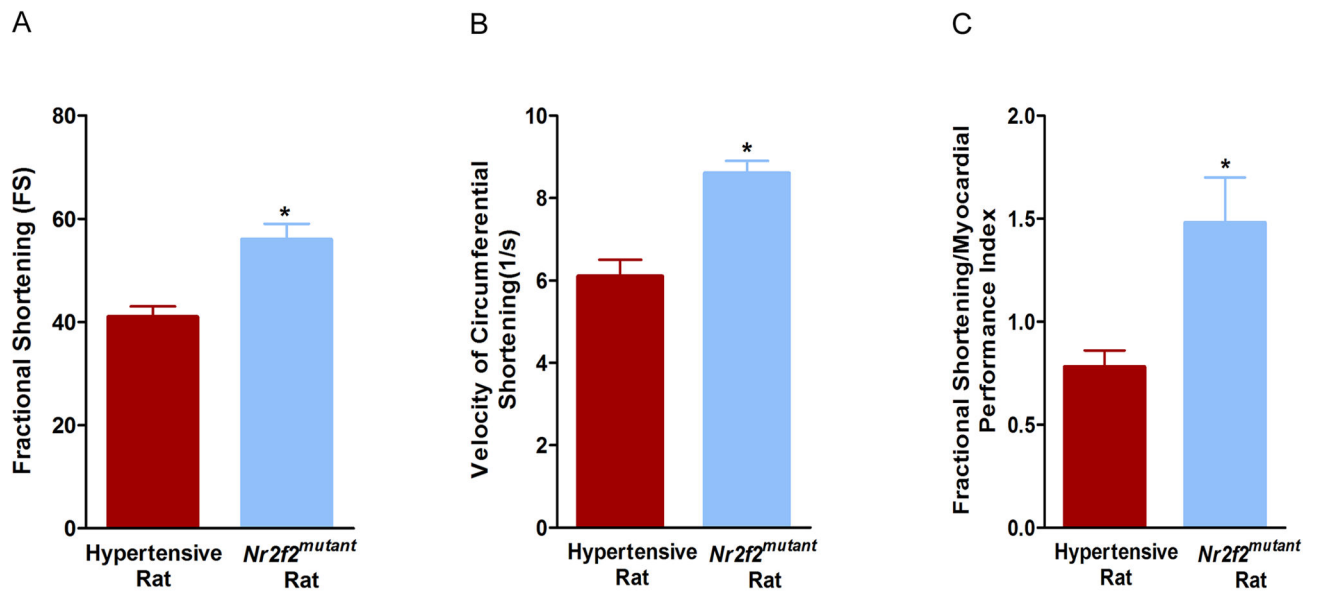


Figure 4. *Nr2f2*^{mutant} rats exhibited superior left ventricular function
Cardiac parameters of male hypertensive rats (n = 12) and *Nr2f2*^{mutant} rats (n = 12) as determined by echocardiography. (A) Fractional Shortening (FS), (B) Velocity of Circumferential shortening (Vcf) (C) Fractional shortening/Myocardial performance index (FS/MPI). Levels of statistical significance for all data were analyzed by independent sample T-Test; *p < 0.05.

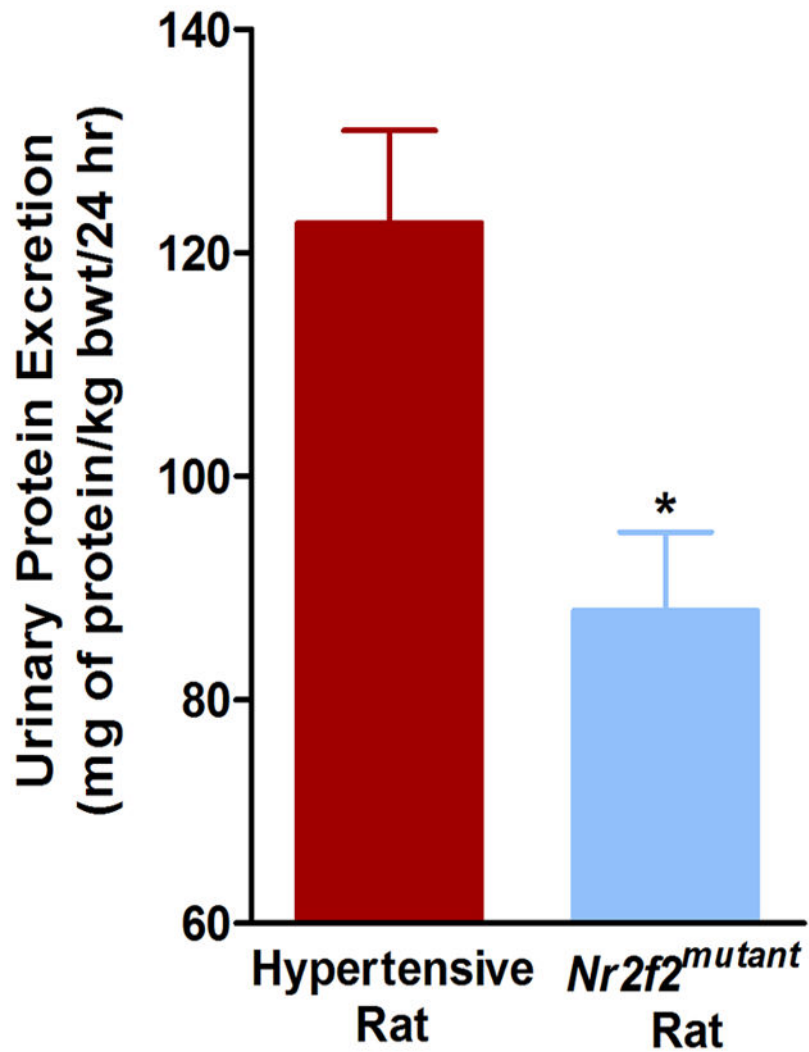


Figure 5. Lower Urinary Protein excretion in *Nr2f2*^{mutant} rats compared with S rats
Total 24 h urine protein was assessed in male hypertensive rats (n = 18) and *Nr2f2*^{mutant} rats (n = 18) as described in the Materials and Methods section. Urinary protein excretion data are presented as milligrams of protein/kg of body weight over a 24h period; t-test, *p<0.05.

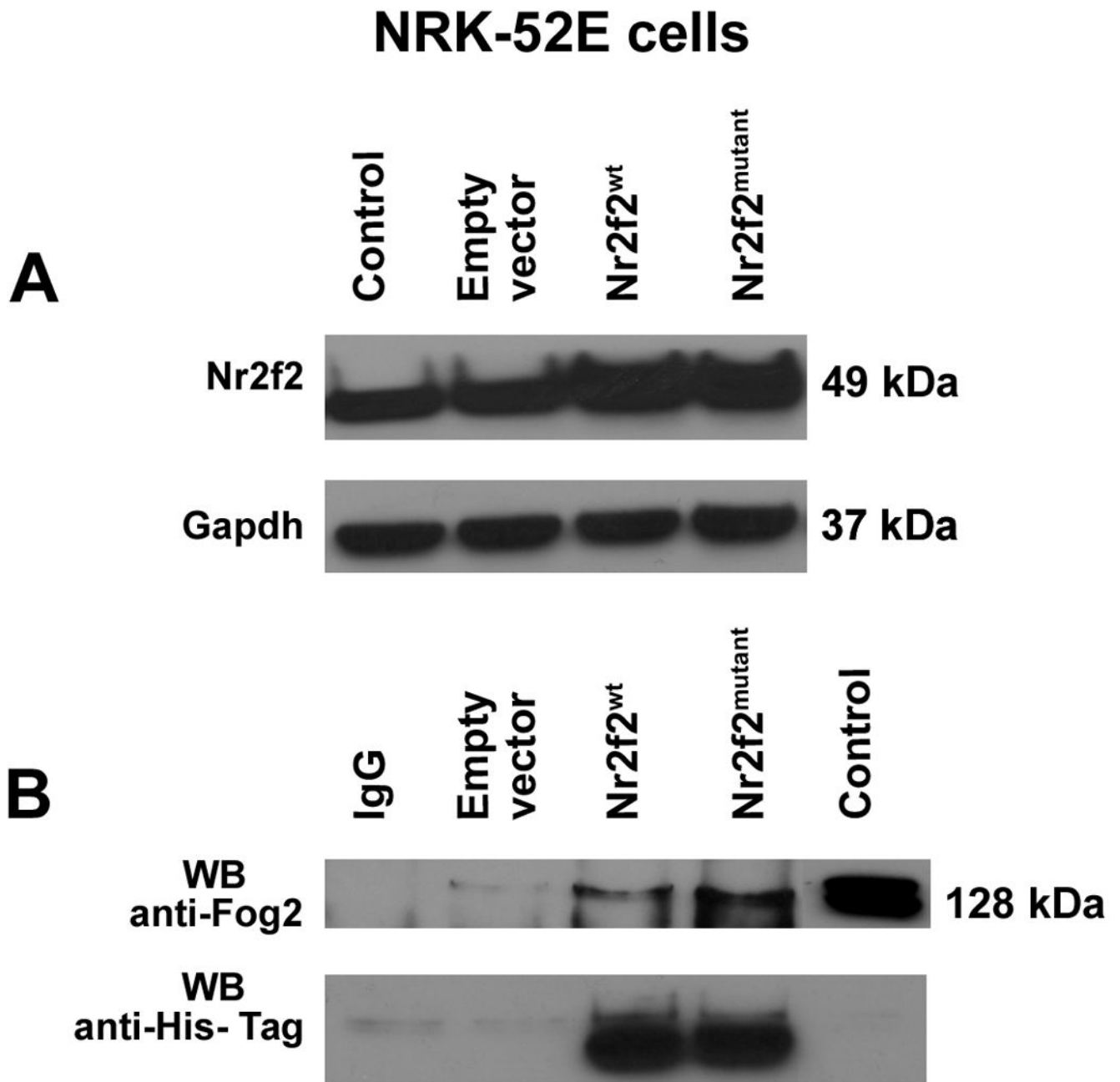


Figure 6. Nr2f2 interaction with Fog2

Co-Immunoprecipitation analysis of Nr2f2 and Fog2 association in NRK52E cells. Extracts from NRK52E cells were transfected with expression plasmid for *Nr2f2^{mutant}* and *Nr2f2^{wildtype}*. (A) Western blotting (WB) of cell lysates for Nr2f2 protein to confirm the over expression of both forms of Nr2f2. Lanes labeled from the left- Control: NRK52E cell lysate; Empty vector: NRK52E cells transfected with empty pcDNA 3.1(-B) myc/His vector; Nr2f2^{wt}: NRK52E cells transfected with Nr2f2 wild type pcDNA 3.1(B) myc/His (-) construct; Nr2f2^{mutant}: NRK52E cells transfected with Nr2f2 mutant pcDNA 3.1(B) myc/His (-) construct. (B) Cell extracts were subjected to immunoprecipitation (IP) with

Nr2f2 antibody (Nr2f2 Ab-1). The immune complexes were captured using Agarose G beads. The immune-precipitated protein samples were denatured, resolved by SDS-Polyacrylamide gel electrophoresis and transferred onto a PVDF membrane. The membrane was probed by western blotting (WB) with anti-Fog2 antibody and anti-His-Tag antibody. A representative of four experiments is shown. Lanes labeled from the left- IgG: IgG antibody control; Empty vector: NRK52E cells transfected with empty pcDNA 3.1(-B) myc/His vector; Nr2f2^{wt}: NRK52E cells transfected with Nr2f2 wild type pcDNA 3.1(B) myc/His (-) construct; Nr2f2^{mutant}: NRK52E cells transfected with Nr2f2 mutant pcDNA 3.1(B) myc/His (-) construct; Control: NRK52E cell lysate. Corroborative data obtained from Histidine-tagged Nr2f2 overexpressed in COS-7 cells are presented in Supplementary Figure 3.

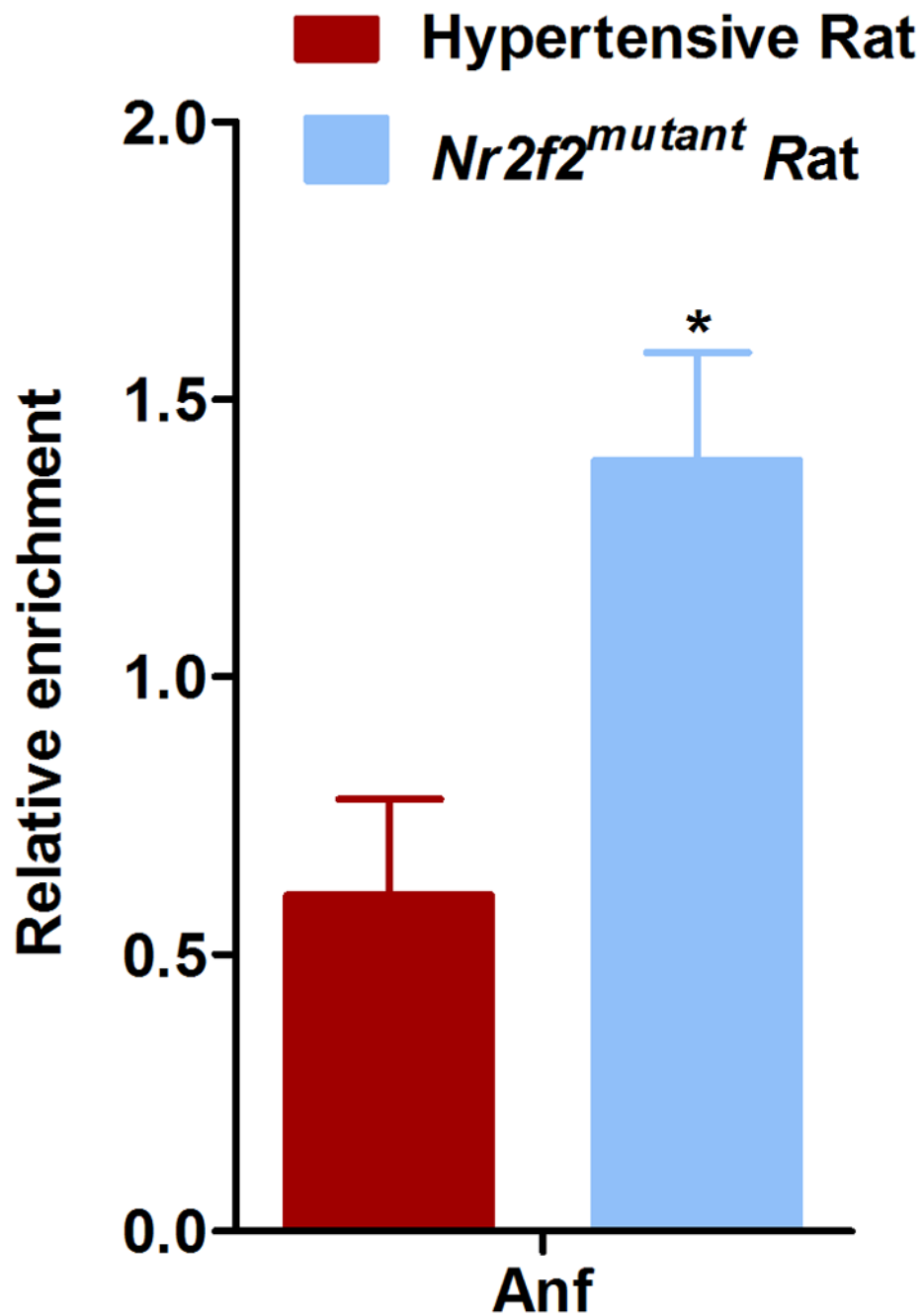


Figure 7. Enrichment of Nr2f2 in Anf promoter region in *Nr2f2*^{mutant} rats

Chromatin immunoprecipitation (ChIP) was conducted using heart tissue lysate from wild-type hypertensive (n=3) and *Nr2f2*^{mutant} rats (n=3) with an anti-Nr2f2 antibody (Nr2f2-Ab1, Abcam) or control IgG antibody (Cell signaling). ChIP enrichment at the Anf locus was quantified by qPCR and normalized to Hemoglobin (*Hbb*) promoter amplification, which was used as a negative control. *p < 0.05.

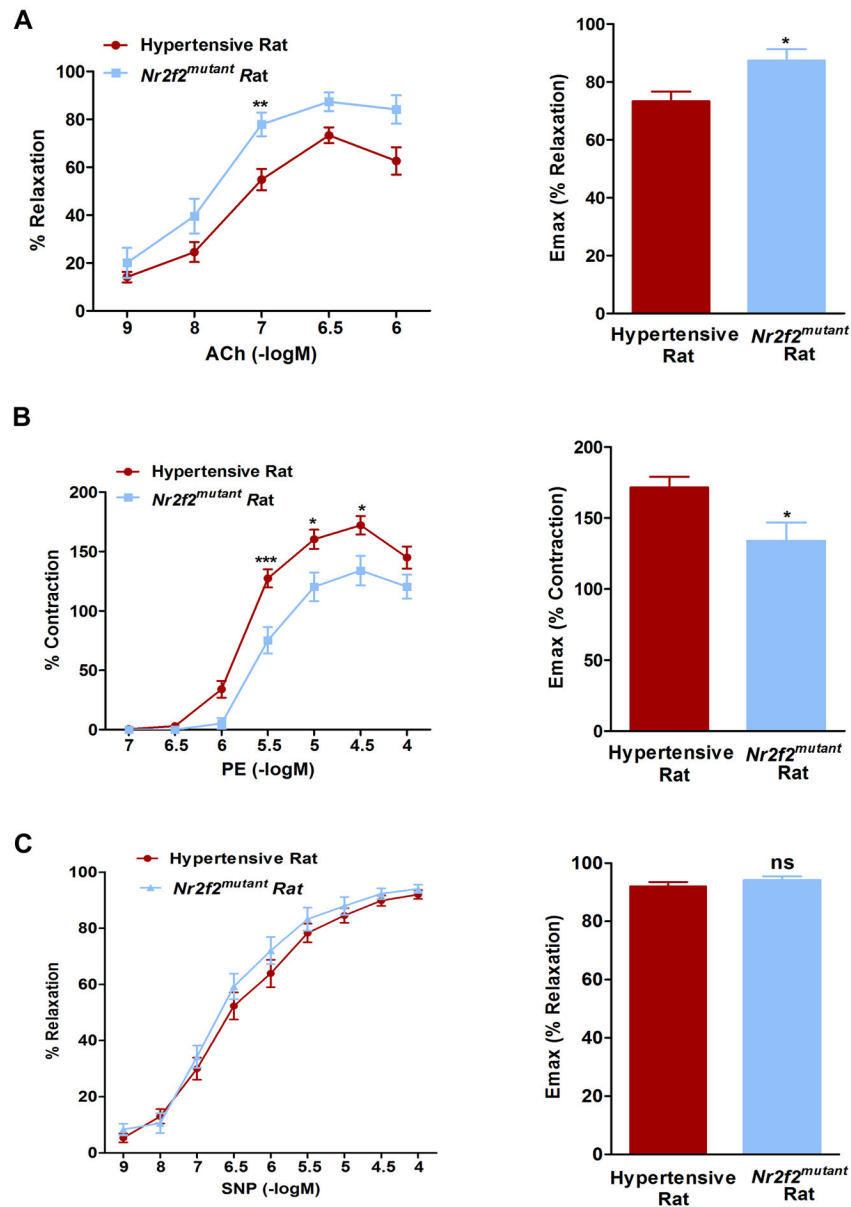


Figure 8. *Nr2f2*^{mutant} rats demonstrated superior vascular function compared with hypertensive rats

(A). Mesenteric arteries from male hypertensive rats (n=9) and *Nr2f2*^{mutant} rats (n=4) were dissected and mounted as described under the methods section. Endothelium-dependent relaxation to Acetylcholine (ACh) was assessed by adding increasing concentrations of ACh (0.001–1 μ M) to the vessel preparation. Relaxation to ACh was expressed as a percentage of the level of pre-constriction induced by submaximal dose of phenylephrine. (B). Cumulative concentration-response curves (CCRC) to phenylephrine (0.1–100 μ M) of mesentery were recorded from wild-type hypertensive (n=9) and *Nr2f2*^{mutant} rats (n=4). Results are expressed as mean \pm S.E. (C). Mesenteric arteries from male hypertensive (n=5) and *Nr2f2*^{mutant} rats (n=5) were dissected and mounted as described under the methods section. Endothelium-independent relaxation to sodium nitroprusside (SNP) was assessed by adding

increasing concentrations of SNP (1nM–100µM) to the vessel preparation. Relaxation to SNP was expressed as a percentage of the level of pre-constriction induced by submaximal dose of phenylephrine. Data were analyzed by t-test. *P<0.05, **P<0.01, ***P<0.001. NS, Not significant.

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