Hypertension

Online Supplements

Pulmonary hypertension-induced GATA4 activation in the right ventricle

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Materials and Methods

Chronic hypoxia treatment

Male Sprague Dawley rats (250 – 300 g) were subjected to chronic hypoxia in a chamber regulated by an OxyCycler Oxygen Profile Controller (BioSpherix, Redfield, NY) that was set to maintain 10% O_2 with influx of N_2. Ventilation was adjusted to remove CO_2, so that its level does not exceed 5,000 ppm. Normoxia controls were subjected to ambient 21% O_2 in another chamber. Animals were fed normal rat chaw during the treatment. In some experiments, rats were subjected to daily i.p. injection with deferoxamine mesylate or MG-132 (Sigma-Aldrich, St. Louis, MO). Georgetown University Animal Care and Use Committee approved all animal experiments, and the investigation conforms to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Histological measurements

For hematoxylin and eosin (H & E) staining, tissues were immersed in buffered 4% paraformaldehyde with 10% sucrose at 4 °C for 24 h, and were embedded in Microtome Tissue Tek II. Frozen tissues were cut to 7-μm-thick slices and mounted on glass slides. Tissue sections were stained with H & E for microscopic evaluation at 200x magnification. Wall thickness values were determined by the IP Lab Software (Scanalytics Inc, VA).

For Verhoff's Van Geison staining and Masson Trichrome staining, tissues were immersed in buffered 10% paraformaldehyde at room temperature, and were embedded in paraffin. Paraffin-embedded tissues were cut and mounted on glass slides. Stained tissue sections were evaluated at 200x magnification.

Electrophoretic mobility shift assays (EMSA)

Nuclear extracts were prepared as described previously.[1,2] For EMSA, binding reactions were performed for 20 min in 5 mmol/L Tris-HCl (pH 7.5), 37.5 mmol/L KCl, 4% (w/v) Ficoll 400, 0.2 mmol/L EDTA, 0.5 mmol/L DTT, 1 μg poly(dI-dC)·poly(dI-dC), 0.25 ng (>20,000 cpm) ^{32}P-labeled double stranded oligonucleotide, and equal protein amounts of nuclear extracts (5 – 10 μg). Electrophoresis of samples through a native 6% polyacrylamide gel was followed by autoradiography. The double stranded oligonucleotide probes containing two GATA consensus elements 5'-CAC TTG ATA ACA GAA AGT GAT AAC TCT-3' (Santa Cruz Biotechnology, Santa Cruz, CA), and 7 regions within the *Gata4* promoter[2] were used. Supershift experiments were performed with 2 μg of antibodies from Santa Cruz Biotechnology. Recombinant human annexin A1 (CalBiochem, San Diego, CA; 10 μg) was included in the binding reaction mixture in some experiments.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA (1 μg) extracted using TRIZOL (Invitrogen, Carlsbad, CA) was reverse-transcribed by oligo(dT) priming and MMLV reverse transcriptase (Applied Biosystems, Foster City, CA). The resultant cDNA was amplified with *Gata4* primers using Taq DNA polymerase (Invitrogen) and resolved on a 1.5% agarose gel containing ethidium bromide.[1]

Western blot analysis

Samples were prepared as previously described.[1,2] Equal protein amounts of samples were electrophoresed through a reducing SDS polyacrylamide gel and electroblotted onto a membrane. The membrane was blocked and incubated with appropriate antibodies (Santa Cruz Biotechnology), and levels of proteins were detected with HRP-linked secondary antibodies and ECL System (Amersham Life Science, Arlington Heights, IL). Carbonylated annexin A1 was measured as previously described.[3]

To precipitate DNA binding proteins, nuclear extracts (100 μg) were incubated with 25 μl of Streptavidin Agarose (Invitrogen) on ice for 1 h and the agarose with non-specifically bound proteins were removed by centrifugation. Supernatant was then incubated with 10 μg of poly(dI-dC)·poly(dI-dC) and 20 μg of biotin-conjugated Probe #2 on ice for 2 h. 25 μl of Streptavidin Agarose were then added and samples were mixed for 1 h at 4°C. Streptavidin Agarose was washed with the buffer 4 times and proteins were collected in Laemmeli buffer by boiling and centrifugation.

Transfection and luciferase assays

HL-1 adult mouse cardiac muscle cells[1,4] were plated in a 12-well plate, and 1 μg DNA/well was transfected using Fugene 6 Transfection Reagent (Roche Diagnostics, Indianapolis, IN) in serum-free, antibiotics-free Claycomb Medium. Co-transfection with the renilla reporter at 6:1 ratio of luciferase to renilla was performed to normalize for transfection efficiency. Cells were transfected for 6 h, and then medium was replaced with FBS-containing Claycomb Medium with antibiotics. Luciferase assays were performed as previously described[2] using the Dual Luciferase Assay kit (Promega, Madison, WI) and a Model TD-20/20 luminometer (Turner Designs, Sunnyvale, CA).

CCAAT box within 250 bp of the *Gata4* promoter region was mutated using following primers: GATAp-F, 5'-AAA CGC GGT ACC AAG GAC GTC GGG CTG–3'(KpnI); GATAp-R, 5'-AAA CGC AAG CTT CTC CGG CTT GTC CCC T–3' (Hind III); CCAAT mut-F, 5'-GTG ACT CCC TTA GTA AAG TCA GCG CAG GCG AT–3'; CCAAT mut-R, 5'-CCT GCG CTG ACT TTA CTA AGG GAG TCA CGT GCA–3'. Underlines indicate restriction digest sites (for GATAp-F and GATAp-R) and mutated nucleotides (for CCAAT mut-F and CCAAT mut-R).

Statistical analysis

Comparisons between 2 groups were analyzed by a two-tailed Student's *t* test, and comparisons between 3 or more groups were analyzed by ANOVA with a Student-Newman-Keuls post-hoc test. $p < 0.05$ was considered to be significant.

References

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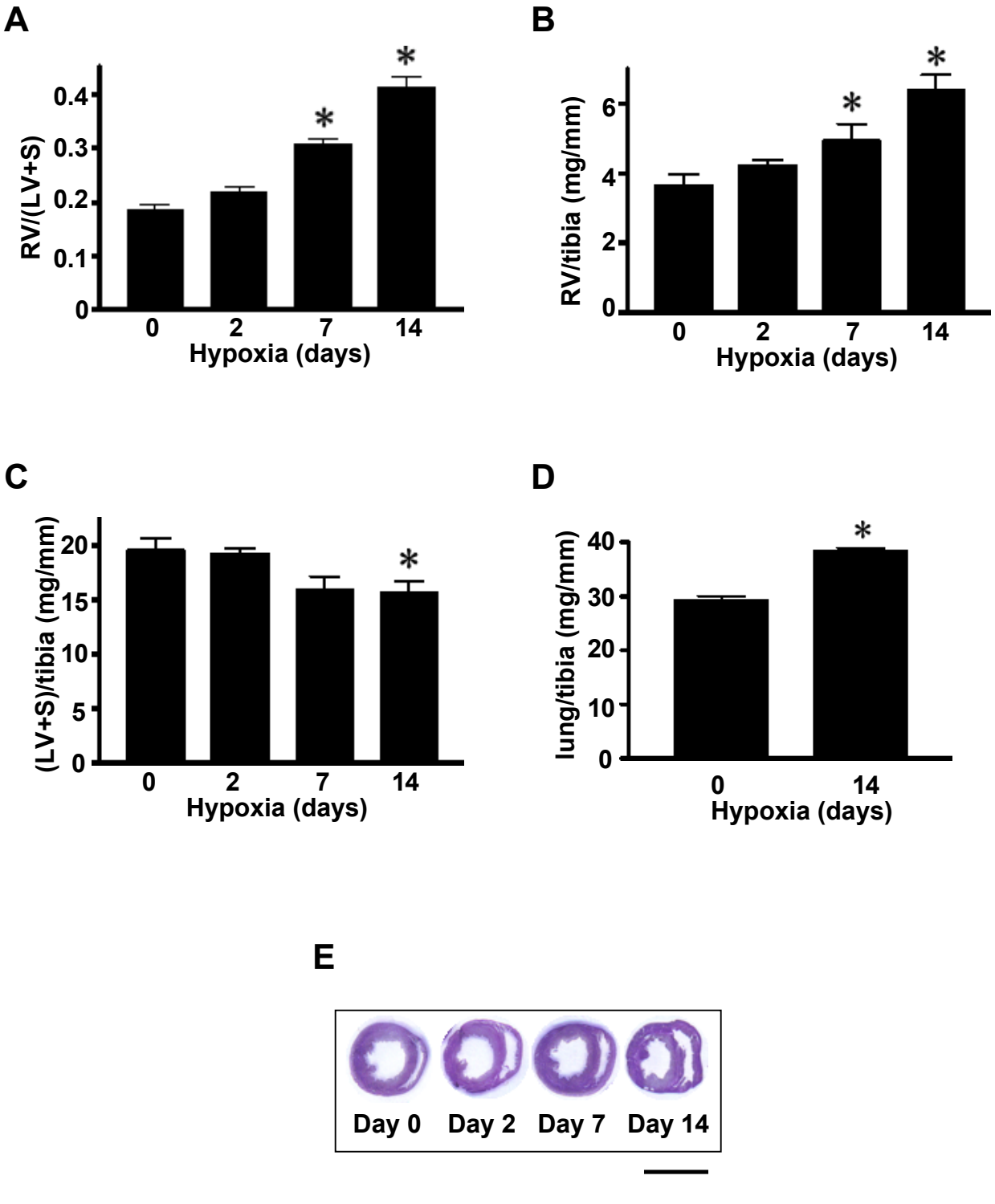
2. Park AM, Nagase H, Vinod Kumar S, Suzuki YJ. Acute intermittent hypoxia activates myocardial cell survival signaling. *Am J Physiol Heart Circ Physiol*. 2007; 292:H751-H757.

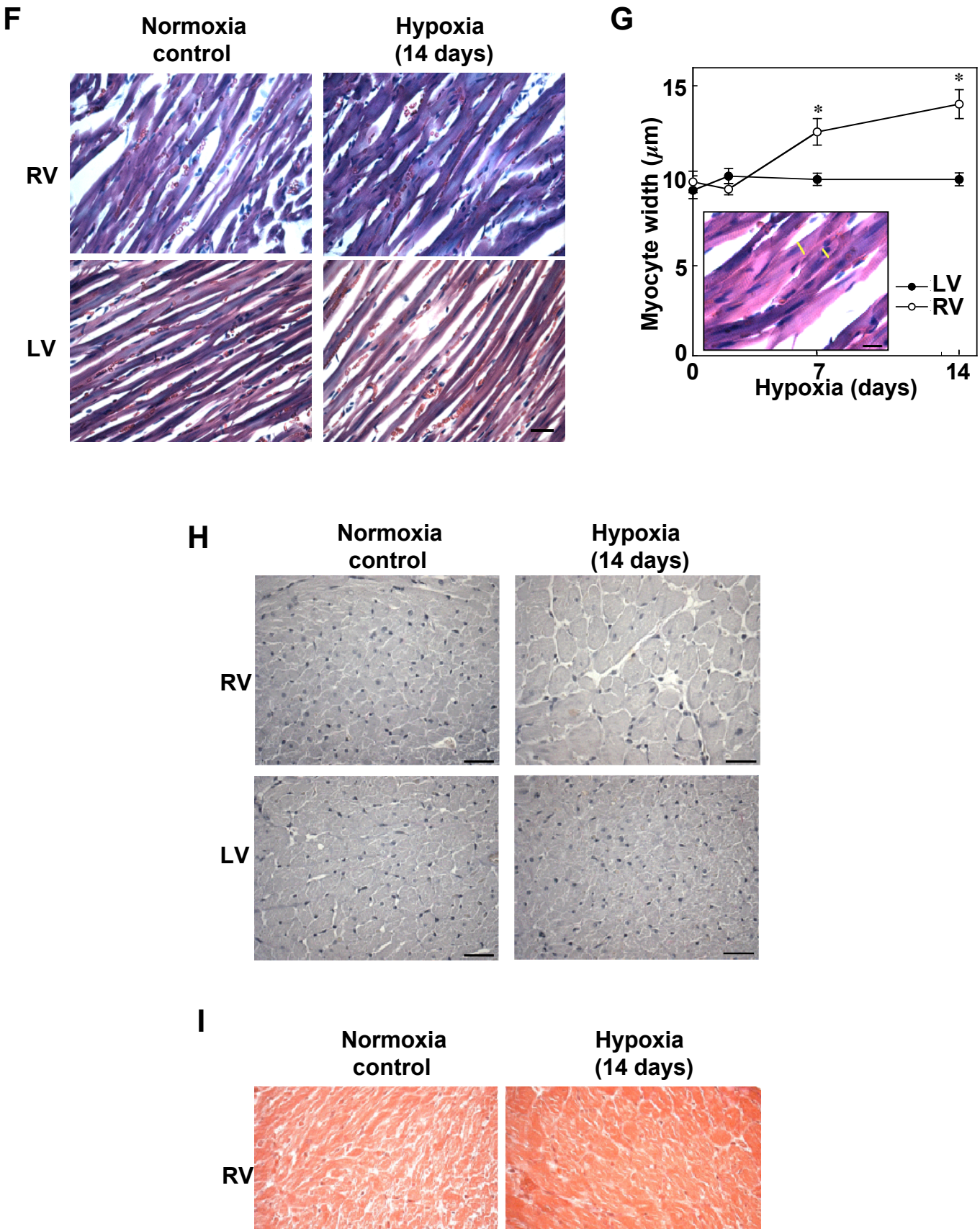
3. Wong CM, Cheema AK, Zhang L, Suzuki YJ. Protein carbonylation as a novel mechanism in redox signaling. *Circ Res*. 2008; 102:310-318.

4. Claycomb WC, Lanson NA Jr, Stallworth BS, Egeland DB, Delcarpio JB, Bahinski A, Izzo NJ Jr. HL-1 cells: a cardiac muscle cell line that contracts and retains phenotypic characteristics of the adult cardiomyocyte. *Proc Natl Acad Sci USA*. 1998; 95:2979-2984.

Park et al. Fig. S1



Park et al. Fig. S1



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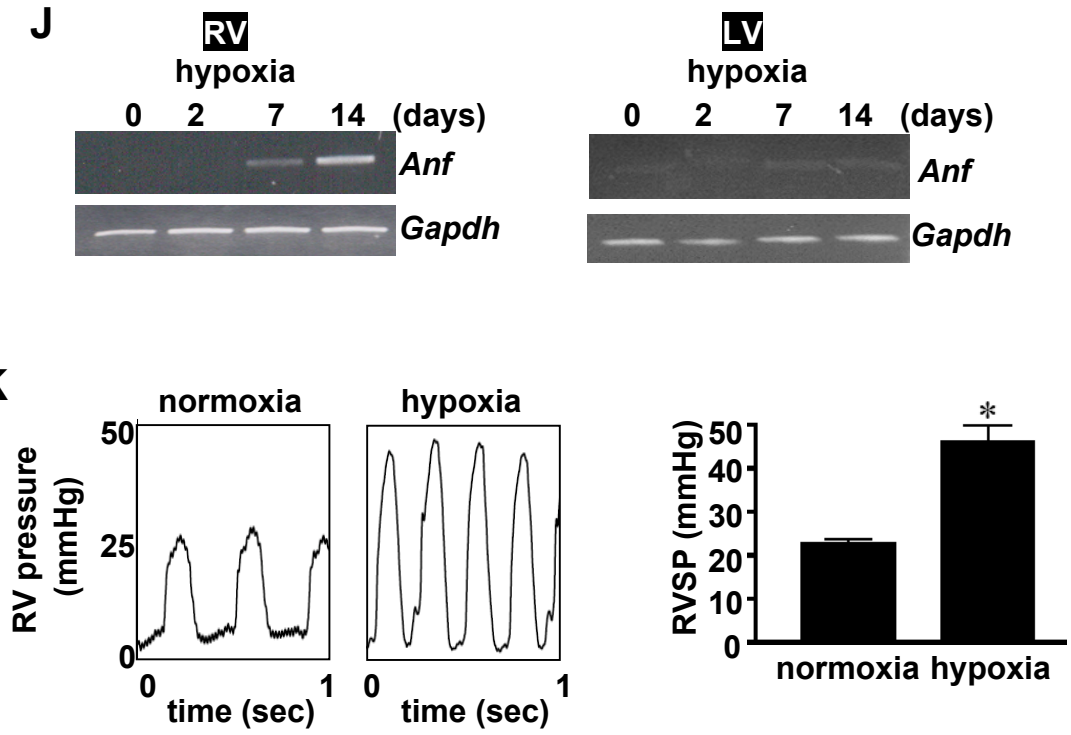
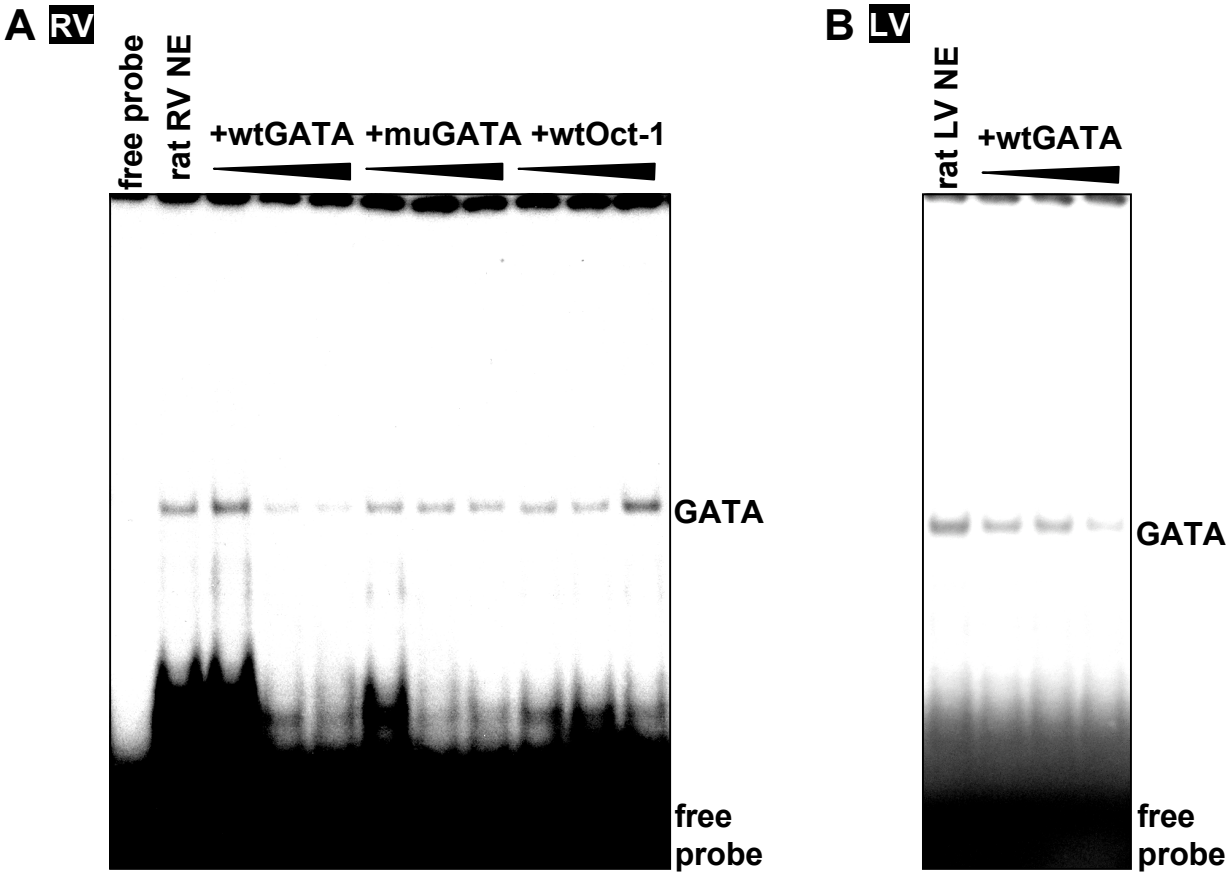


Fig. S1: Hypoxic pulmonary hypertension induces right ventricular (RV) hypertrophy. Rats were subjected to chronic hypoxia at 10% O_2 for durations indicated. Tibia lengths were measured; right ventricle (RV), left ventricle (LV) + septum (S) and lungs were dissected and weighed; and (A) RV weight/(LV + S weight), (B) RV weight/tibia length, (C) (LV + S weight)/tibia length and (D) lung weight/tibia length were calculated. Bar graphs represent means ± SEM (n = 6). (E) H & E staining of the heart demonstrating progressively increased RV thickness in response to chronic hypoxia. Size marker: 1 cm. (F) Magnified views of H & E stained heart showing thickened myocytes in the RV, but not in the LV, of hypoxia-treated rats. Size marker: 20 μm. (G) Cardiac myocyte thickness (as indicated by lines within the image) was quantitatively measured in randomly chosen 10 cells per group in the H & E stained specimen using IPLab Imaging Software. The line graph represents means ± SEM (n = 10). Size marker in the representative image: 20 μm. (H) Verhoff's Van Geison staining of the heart demonstrating thickened myocytes in RV, but not in the LV, of hypoxia-treated rats. Size marker: 20 μm. (I) Masson Trichrome staining, showing no fibrosis in concentrically hypertrophied RV in response to chronic hypoxia. Size marker: 20 μm. (J) RNA was isolated from RV and LV, and atrial natriuretic factor (*Anf*) mRNA and glycealdehyde 3-phosphate dehydrogenase (*Gapdh*) mRNA (as control) were measured by RT-PCR. (K) RV pressure was monitored by inserting a Millar catheter (1.4 F) to the apex in anaesthetized and ventilated rats using PowerLab 8/30 High Performance Data Acquisition System and Chart Pro Software (ADInstruments). Bar graphs represent means ± SEM (n = 3) of right ventricular systolic pressure (RVSP). (*) denotes values significantly different from the normoxia control (0 day hypoxia) values at $p < 0.05$.

Park et al. Fig. S2



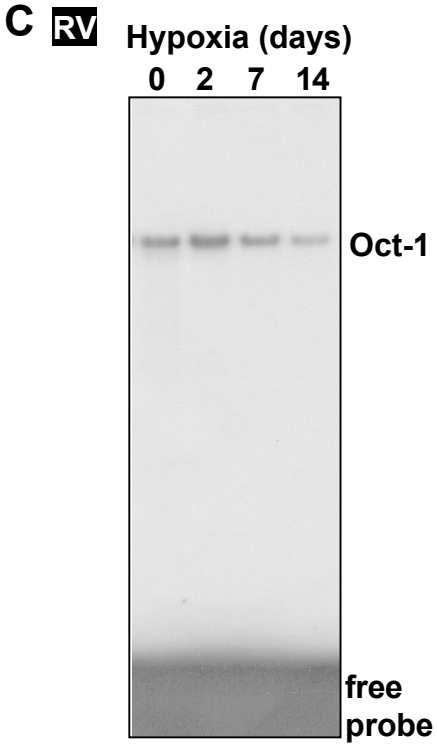


Fig. S2: Control experiments for electrophoretic mobility shift assays (EMSA). (A) Rat right ventricular (RV) nuclear extracts were subjected to EMSA in the presence of cold oligonucleotide competitors with the GATA consensus sequence TGATAA (wtGATA), a mutated GATA sequence TCTTAA (muGATA), or unrelated Oct-1 binding sequence (wtOct-1). Increasing amounts (0.5, 1 and 2 ng) of cold competitors were used. (B) Rat left ventricular (LV) nuclear extracts were subjected to EMSA in the presence of a cold oligonucleotide competitor with the GATA consensus sequence. (C) Rats were subjected to chronic hypoxia at 10% O_2 for durations indicated. RV nuclear extracts were subjected to EMSA to monitor Oct-1 DNA binding activity as a control, which does not get activated in response to chronic hypoxia in association with the activation of GATA-4.

Park et al. Fig. S3

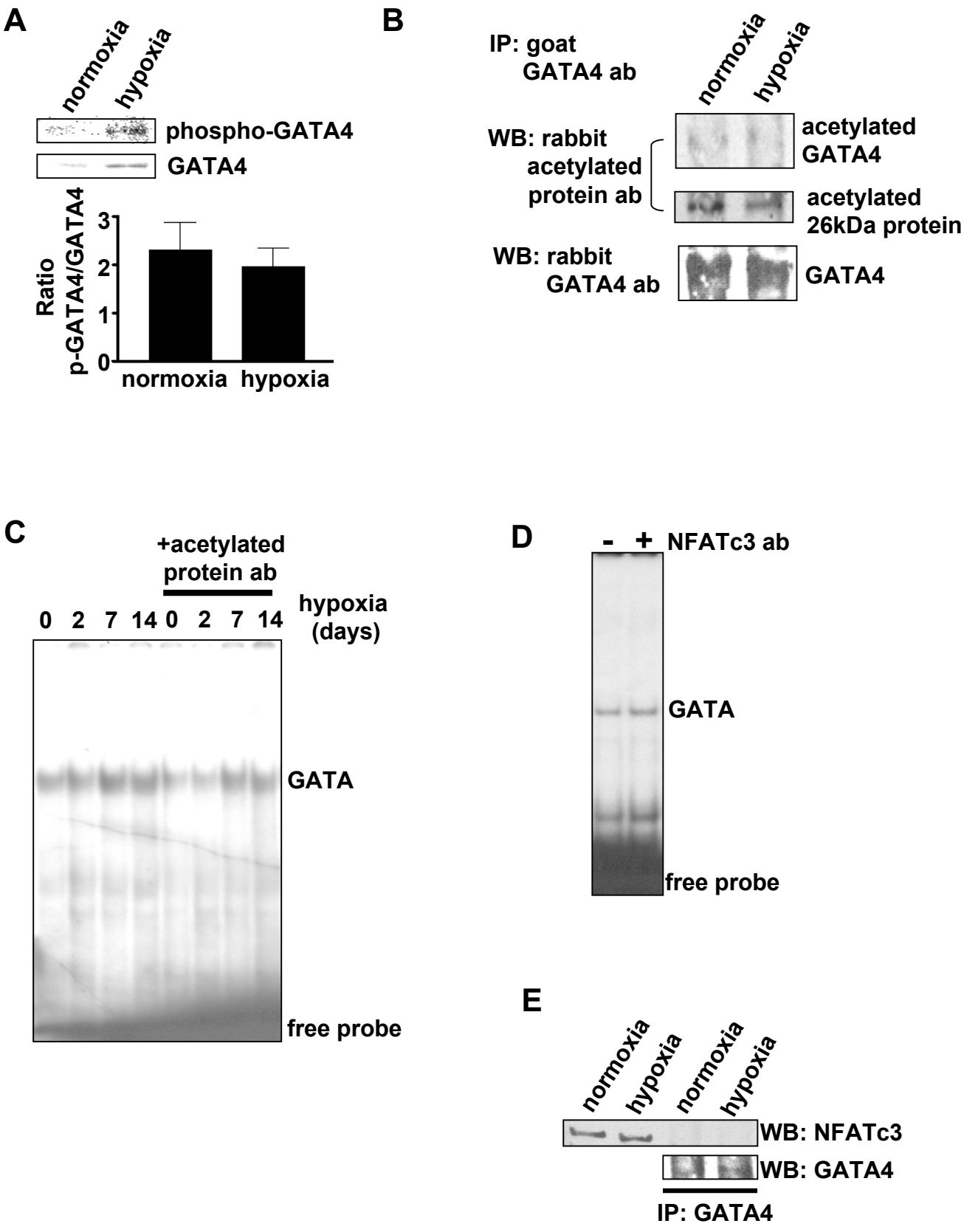


Fig. S3: Effects of hypoxic pulmonary hypertension on post-translational modification mechanisms of GATA4 in RV. (A) Rats were subjected to chronic hypoxia or normoxia for 14 days. Levels of phosphorylated GATA4 (phospho-GATA4) and GATA4 protein expression were monitored in RV nuclear extracts by Western blotting (n = 3). Bar graph represents means ± SEM of the ratio of phosphorylated GATA4 and GATA4 protein expression levels. No significant differences were noted between normoxia and hypoxia. (B) Rats were subjected to chronic hypoxia or normoxia for 14 days. RV nuclear extracts were immunoprecipitated with goat GATA4 antibody, followed by Western blotting with rabbit acetylated protein antibody. Acetylated GATA4 was not detected, while acetylation of other proteins (such as 26 kDa protein) was measurable. The membranes were also blotted with the rabbit GATA4 antibody to show the success of immunoprecipitation for GATA4. (C) Rats were subjected to chronic hypoxia for durations indicated. RV nuclear extracts were subjected to EMSA in the absence and presence of acetylated protein antibody. The GATA band was not influenced by this antibody. (D) RV nuclear extracts from rats treated with chronic hypoxia for 14 days were subjected to EMSA in the absence and presence of NFATc3 antibody. The GATA band was not influenced by this antibody. (E) RV nuclear extracts from rats treated with normoxia or chronic hypoxia for 14 days were immunoprecipitated with GATA4 antibody, followed by Western blotting with NFATc3 or GATA4 antibody. No NFAT-GATA4 interactions were promoted by chronic hypoxia. Left two lanes show control Western blot experiments without immunoprecipitation, showing that rat RV does express NFATc3 protein.

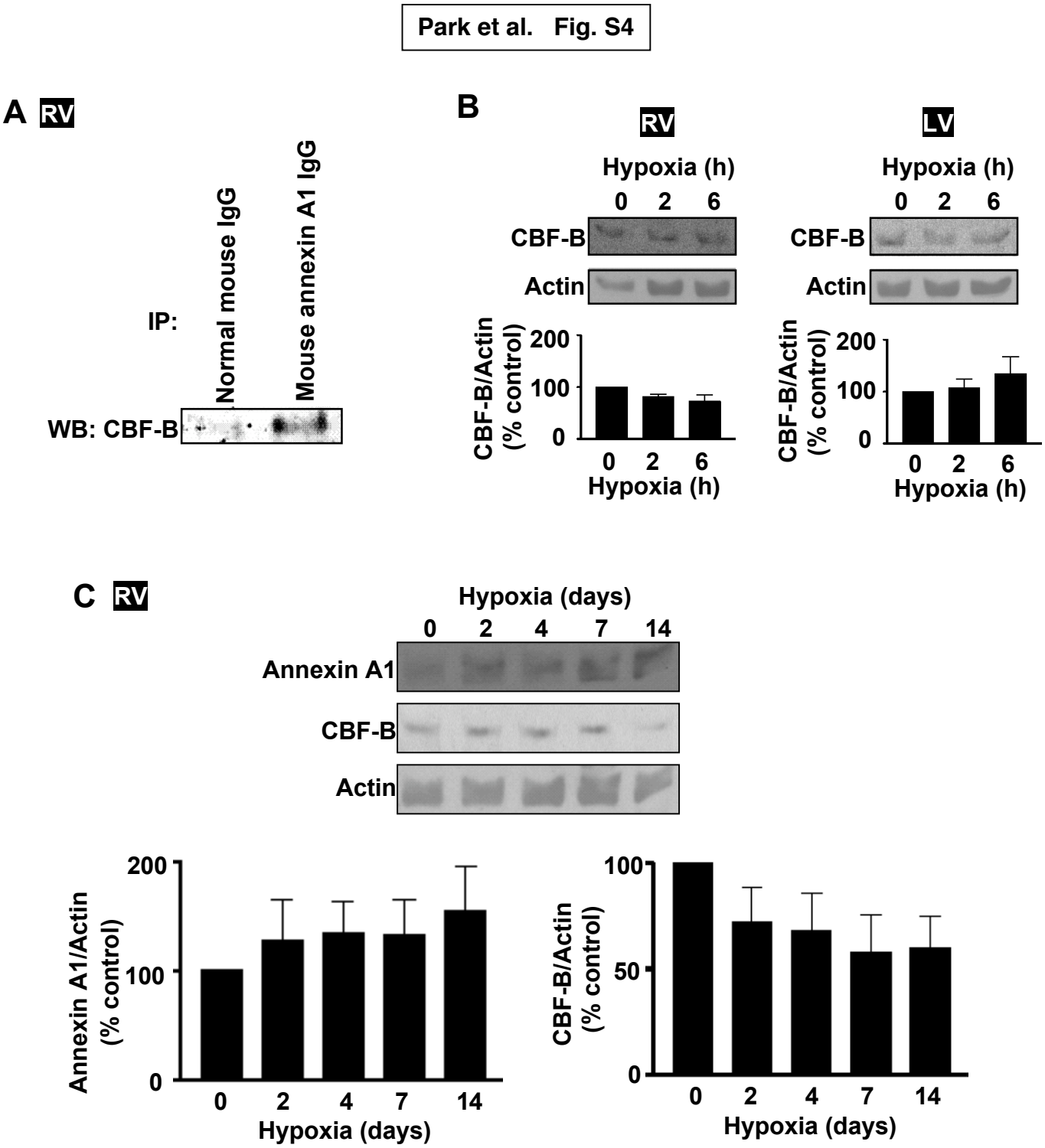


Fig. S4: Control experiments concerning CBF/NF-Y and annexin A1. (A) Annexin A1 interactions with CBF transcription factor. Rat RV nuclear extract (NE) samples were immunoprecipitated with mouse annexin A1 antibody or normal mouse IgG control as indicated and immuno-blotted with the goat CBF-B antibody. (B) Rats were subjected to sustained hypoxia for durations indicated. CBF-B and actin protein levels were monitored by Western blotting. Bar graphs represent means ± SEM of % control of the ratio of CBF-B to actin (n = 4). (C) Rats were subjected to chronic hypoxia for durations indicated. Annexin A1, CBF-B and actin protein levels were monitored by Western blotting. Bar graphs represent means ± SEM (n = 5).

Park et al. Fig. S5

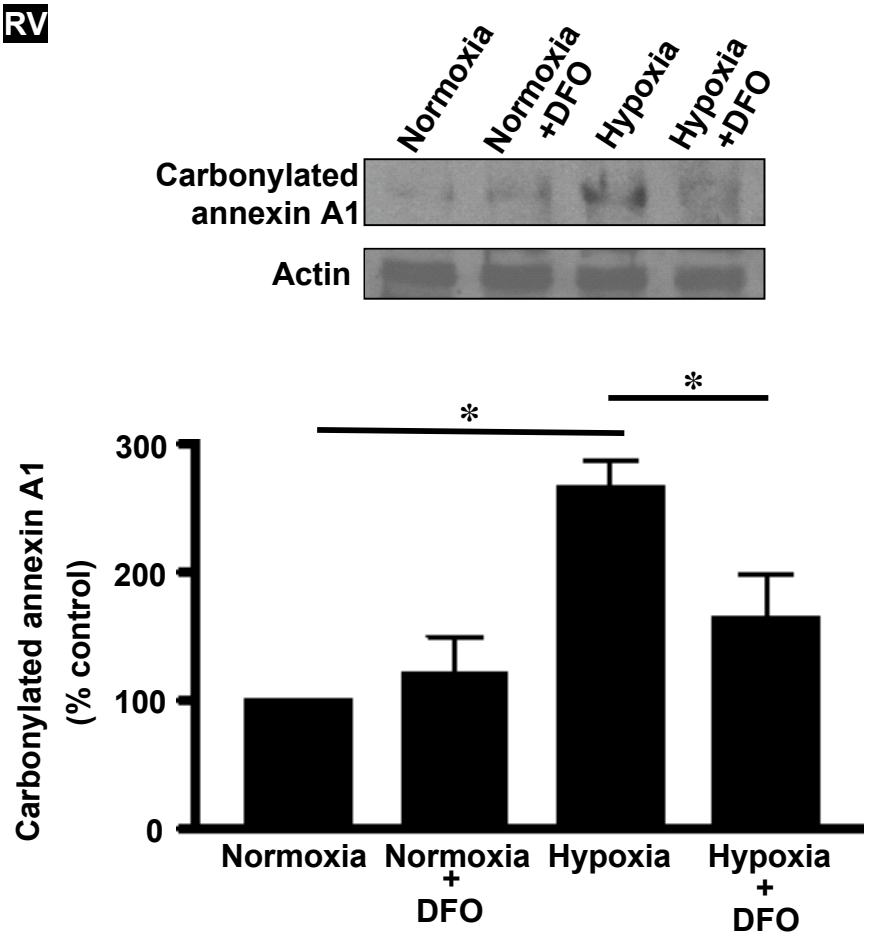


Fig. S5: Effects of deferoxamine (DFO) on annexin A1 carbonylation. Rats were injected (i.p.) with DFO (20 mg/kg body weight) or saline, then subjected to hypoxia at 10% O_2 for 2 h. RV homogenates were derivatized with dinitrophenylhydrazine (DNPH), immunoprecipitated with the antibody for DNPH-derivatized proteins, and subjected to SDS-PAGE and immunoblotting with the annexin A1 antibody. Values in the bar graph represent means ± SEM (n = 6). (*) denotes values significantly different at $p < 0.05$.

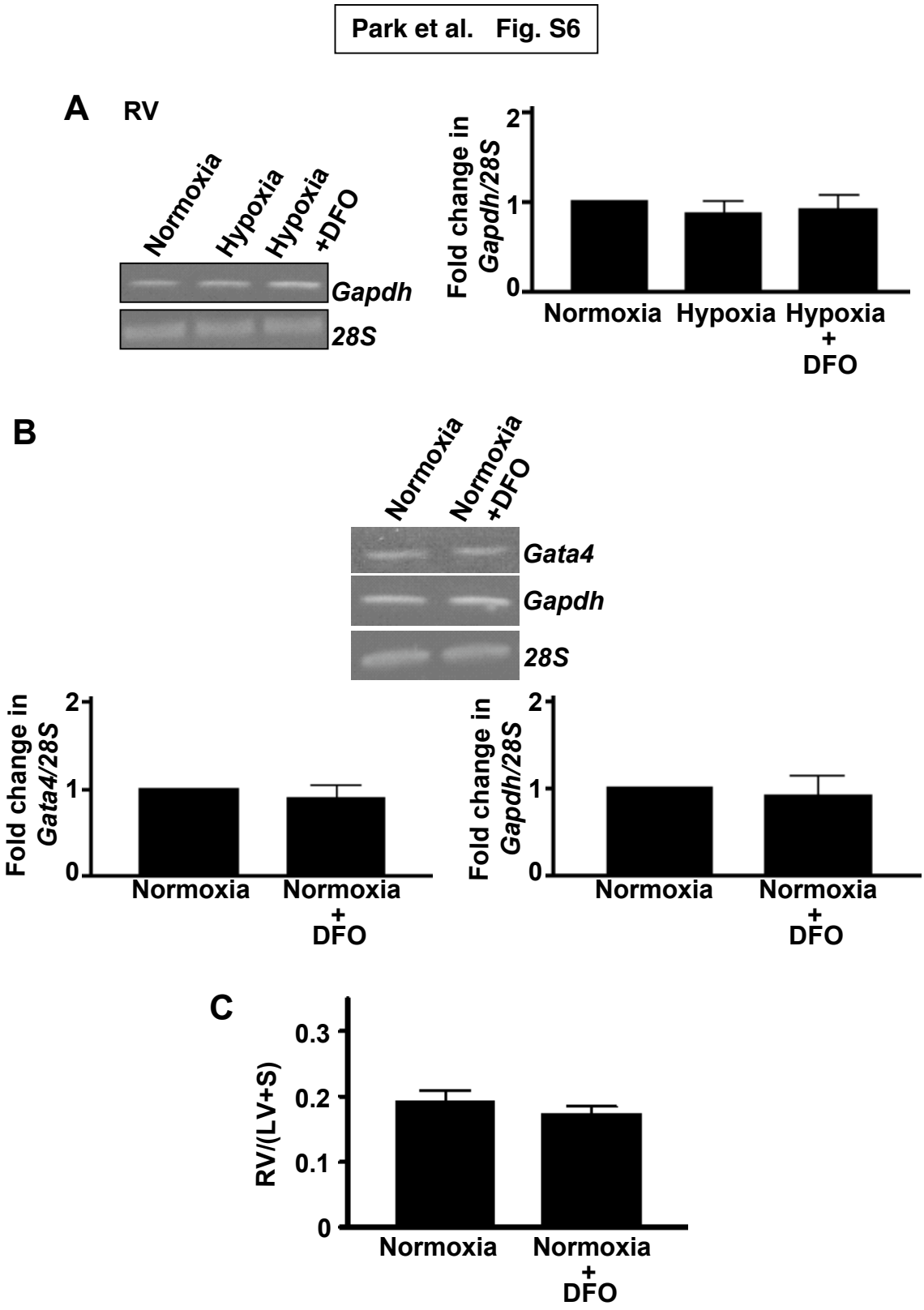


Fig. S6: Control experiments on effects of deferoxamine (DFO). (A) Rats were injected (i.p.) with DFO (20 mg/kg body weight) or saline daily during the 4 day exposure to hypoxia at 10% O_2. *Gapdh* mRNA and *28s* rRNA levels were monitored by RT-PCR. (B) DFO or saline was injected to normoxic control rats. *Gata4* mRNA, *Gapdh* mRNA and *28s* rRNA levels were monitored by RT-PCR. (C) Masses of RV, LV and septum (S) were measured and RV/(LV+S) values were calculated as an estimate of RV hypertrophy. Values in the bar graph represent means ± SEM (n = 4).

Park et al. Fig. S7

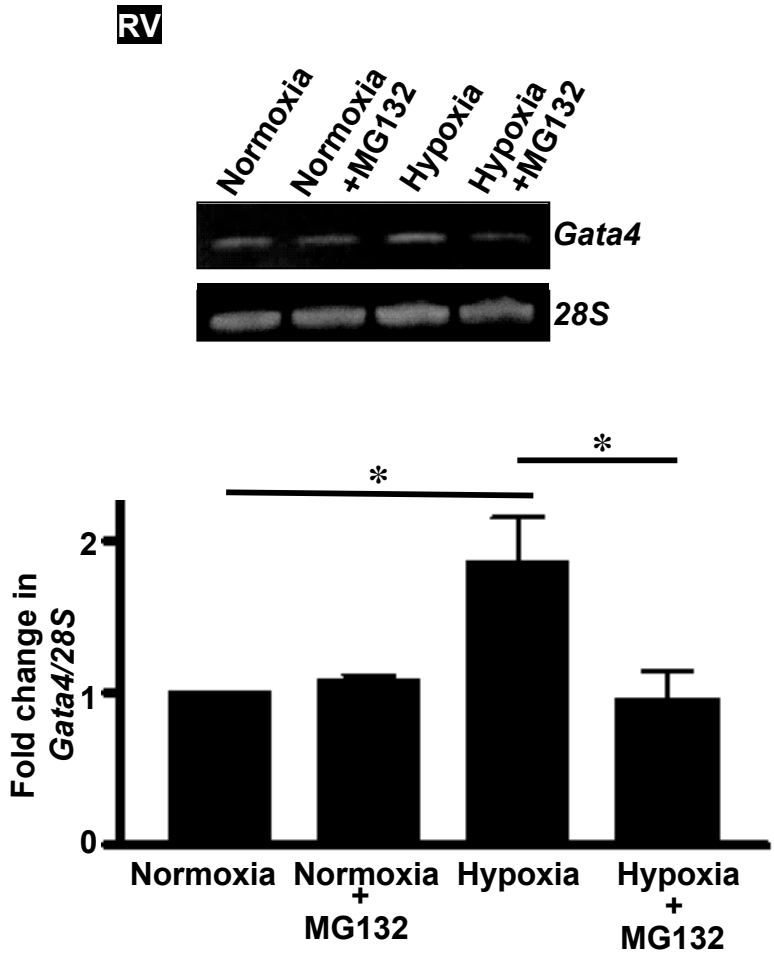


Fig. S7 Effects of MG132 on hypoxic pulmonary hypertension-induced upregulation of *Gata4* expression in RV. Rats were injected (i.p.) with MG132 (10 mg/kg body weight) and exposed to hypoxia at 10% O_2 for 4 days. RNA was isolated from RV, and *Gata4* mRNA and *28s* rRNA levels were monitored by RT-PCR. Values in the bar graph represent means ± SEM (n = 4). (*) denotes values significantly different at $p < 0.05$.

Park et al. Fig. S8

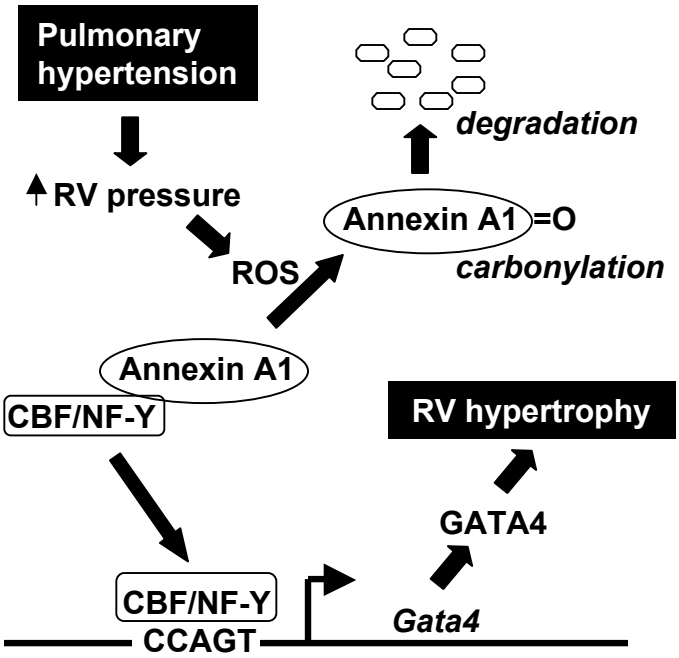


Fig. S8 Proposed mechanism for GATA4 activation in the right ventricle (RV) in response to pulmonary hypertension. Pulmonary hypertension exerts pressure overload to the RV, resulting in the generation of reactive oxygen species (ROS), which in turn carbonylate annexin A1 that is bound to CBF/NF-Y transcription factor. Carbonylated annexin A1 is degraded, resulting in liberated CBF/NF-Y that can bind to CCAAT box within the *Gata4* promoter. CBF/NF-Y binding enhances gene transcription of *Gata4* and increases the level of GATA4 transcription factor, which in turn promotes gene expression of hypertrophic regulators.