Pulmonary Hypertension-Induced GATA4 Activation in the Right Ventricle

Ah-Mee Park, Chi-Ming Wong, Ludmila Jelinkova, Lingling Liu, Hiroko Nagase, Yuichiro J. Suzuki

Abstract—The major cause of death among pulmonary hypertension patients is right heart failure, but the biology of right heart is not well understood. Previous studies showed that mechanisms of the activation of GATA4, a major regulator of cardiac hypertrophy, in response to pressure overload are different between left and right ventricles. In the left ventricle, aortic constriction triggers GATA4 activation via posttranslational modifications without influencing GATA4 expression, while pulmonary artery banding enhances GATA4 expression in the right ventricle. We found that GATA4 expression can also be increased in the right ventricle of rats treated with chronic hypoxia to induce pulmonary hypertension and investigated the mechanism of increased GATA4 expression. Examination of Gata4 promoter revealed that CCAAT box plays an important role in gene activation, and hypoxic pulmonary hypertension promoted the binding of CCAAT-binding factor/nuclear factor-Y (CBF/NF-Y) to CCAAT box in the right ventricle. We found that CBF/NF-Y forms a complex with annexin A1, which inhibits DNA binding activity. In response to hypoxic pulmonary hypertension, annexin A1 gets degraded, resulting in CBF/NF-Y-dependent activation of Gata4 gene transcription. The right ventricle contains a higher level of CBF/NF-Y compared to the left ventricle, and this may allow for efficient activation in response to annexin A1 degradation. Signaling via iron-catalyzed protein oxidation mediates hypoxic pulmonary hypertension-induced annexin A1 degradation, Gata4 gene transcription, and right ventricular hypertrophy. These results establish a right heart-specific signaling mechanism in response to pressure overload, which involves metal-catalyzed carboxylation and degradation of annexin A1 that liberates CBF/NF-Y to activate Gata4 gene transcription. (Hypertension. 2010;56:1145-1151.)

Key Words: heart failure ■ hypertension ■ hypertrophy ■ hypoxia ■ pressure overload ■ pulmonary hypertension ■ redox signaling

Pulmonary hypertension (PH) is characterized by increased pulmonary arterial pressure and vascular resistance, which interfere with the ejection of blood by the right ventricle (RV) and ultimately causes right heart failure. While the major cause of death among PH patients is RV failure, the RV biology has not been well defined.1

GATA4 regulates transcription of genes that are expressed during adult clinical cardiac hypertrophy.2 Transgenic mice with cardiac-specific overexpression of GATA4 exhibit concentric hypertrophy of atria as well as RV and left ventricle (LV).3

While similarities and differences between adult LV and RV have not been well characterized, these 2 ventricles originate from different precursors.4 Furthermore, in mouse embryo, knocking out Gata4 induced RV hypoplasia.5

Previous studies indicated that mechanisms of GATA4 activation are different between adult LV and RV. Pressure overload via aortic constriction increased GATA4 activity without enhancing GATA4 expression in LV.6 In contrast, pressure overload via pulmonary artery banding enhanced GATA4 expression in RV.7 Similarly, we found that increased pulmonary arterial pressure by chronic hypoxia (CH) increased GATA4 expression. The present study examined the mechanism of enhanced GATA4 gene expression by CH-mediated PH in RV.

Materials and Methods
See online supplemental material at http://hyper.ahajournals.org.

Results
Effects of Hypoxic PH on GATA4 in RV
Adult rats were subjected to CH at 10% O2 for 24 hours per day for 2, 7, and 14 days.8 CH promoted RV hypertrophy as indicated by RV/(LV+S) mass ratio (Figure S1A) as well as RV mass/tibia length ratio (Figure S1B). RV mass became significantly higher compared with control rats by 7 days of hypoxia and progressively increased. No increases in LV and septum masses were noted (Figure S1C). Lung mass was increased, consistently with the occurrence of pulmonary

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vascular thickening (Figure S1D). Histological analyses of the heart showed time-dependent thickening of the RV wall (Figure S1E), indicating the occurrence of concentric hypertrophy. Microscopic analysis of the hematoxylin and eosin–stained heart revealed increased RV myocyte thickness in animals treated with hypoxia, but LV myocytes were unchanged (Figure S1F). Quantitative analysis showed a significant increase in RV myocyte thickness after 7 days of hypoxia (Figure S1G). Verhoff’s Van Geison staining confirmed the occurrence of concentric RV muscle hypertrophy (Figure S1H), without notable fibrosis as monitored by Masson trichrome staining (Figure S1I). Increased diastolic RV wall thickness in response to 2 weeks of CH was observed by 2-dimensional echocardiography using a VisualSonics High-Resolution In Vivo Imaging System (0.50 mm normoxia versus 1.03 mm hypoxia). Expression of fetal genes that are often used as markers for ventricular hypertrophy such as atrial natriuretic factor (Anf) were induced in hypertrophied RV, but not in LV (Figure S1J). Hemodynamic measurements using a Millar catheter demonstrated that CH increased RV systolic pressure (Figure S1K).

Results from electrophoretic mobility shift assays (EMSAs) demonstrated that CH-induced RV hypertrophy was associated with GATA4 activation in RV (Figure 1A) but not in LV (Figure 1B). The increase was apparent after 2 days of hypoxia, and significant increases were noted at 7 and 14 days of hypoxia (Figure 1C). GATA binding activity in RV was reduced by the cold competitor with GATA consensus sequence TGATAA (wtGATA), but not by mutant TCTTAA (muGATA) or unrelated Oct-1 binding sequence (wtOct-1) (Figure S2A). GATA binding activity in LV was also reduced by the cold competitor (Figure S2B). Supershift experiments confirmed that GATA activity is due to GATA4 in RV (Figure 1A) and LV (Figure 1B). Hypoxia did not increase the DNA binding activity of Oct-1 (Figure S2C). Increased GATA4 DNA binding activity in RV by CH was associated with increased GATA4 protein expression (Figure 1D).

Consistent with the earlier study of pulmonary artery banding,7 induction of PH by CH also increased Gata4 mRNA expression in RV, but not in LV (Figure 2A). In these experiments, we added earlier time points and found that a significant increase in Gata4 mRNA was detected as early as 6 hours of sustained hypoxia and maintained for at least for 2 weeks. We did not detect the occurrence of known posttranslational modification mechanisms for GATA4 such as phosphorylation9,10 (Figure S3A), acetylation11,12 (Figure S3B and S3C), and interactions with other transcription factors such as nuclear factor of activated T-cells13 (Figure S3D and S3E).

**Studies of Gata4 Promoter**

We hypothesized that Gata4 gene transcription is activated by hypoxic PH in RV. We previously found that the 250-bp region immediately upstream from the transcriptional start site of mouse Gata4 gene contains key regulatory elements14,15 To test whether this region is influenced by CH in RV, EMSA probes were constructed by dissecting this

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**Figure 1.** Hypoxic PH promotes GATA4 activity and expression in the RV. A, B, Rats were subjected to CH. Nuclear extracts from RV (A) and LV (B) were subjected to EMSA to monitor GATA DNA binding activity in the absence or presence of antibodies (Ab). ss indicates supershifted bands. C, Values represent means±SEM. D, GATA4 protein levels in nuclear extracts were measured by Western blot. A nonspecific (NS) band suggests no differences in loading. Asterisks indicate significant differences from normoxia control (n=5 to 6).
250-bp region into 7 subregions.\textsuperscript{14} EMSA with RV nuclear extracts showed DNA-binding proteins, which interacted with these probes (Figure 2B). While binding patterns of other probes were unaltered, using probe 2, we identified a band that is increased in response to CH in RV (Figure 2C). Time-course studies revealed that probe 2 binding activity was significantly increased by CH in RV as early as 6 hours and remained increased up to 14 days. No activation of probe 2 binding was noted in LV (Figure 2C).

Role of CCAAT Box
Probe 2 contains CCAAT box as depicted by the sequence CCAGT in Figure 3A. Mutation (Figure 3B) or truncation (Figure 3C) of CCAAT box eliminated the band that was affected by CH. A cold competitor containing CCAAT box effectively eliminated binding of the radiolabeled probe (Figure 3D). To test whether CCAAT box plays a functional role in gene transcription, we mutated the CCAAT box site (GCCAGT to TAAAGT) and constructed a luciferase vector. Transfection of HL-1 cells demonstrated that this mutation significantly attenuated the transcriptional activity of the 250-bp \textit{Gata4} promoter (Figure 3E). These results suggest that proteins, which bind to CCAAT box within probe 2, are affected by CH.

Role of CCAAT-Binding Factor/Nuclear Factor-Y
To identify the transcription factor(s) that comprises the CCAAT box binding complex, we performed supershift experiments with antibodies against factors that are known to bind to CCAAT box, including C/EBP-\( \beta \), CCAAT-binding factor/nuclear factor-Y (CBF/NF-Y), and nuclear factor-1.\textsuperscript{16} The antibody against CBF-B (a subunit of CBF/NF-Y) eliminated the band that is affected by CH (Figure 4A), possibly because of the interference of the DNA binding activity by the antibody. This effect was not observed with antibodies for C/EBP-\( \beta \) (Figure 4A) or nuclear factor-1 (data not shown). Furthermore, experiments in which CCAAT box binding proteins were pulled down with biotinylated probe 2 and streptavidin-agarose showed this complex contained CBF/NF-Y (Figure 4B). Binding of CBF/NF-Y toward CCAAT box within probe 2 of the \textit{Gata4} promoter was also observed in chromatin isolated from fixed RV tissues of CH-treated rats using the chromatin immunoprecipitation assay (data not shown). To provide direct evidence that CBF/NF-Y plays a role in the regulation of \textit{Gata4} gene transcription, HL-1 cells were subjected to small interfering RNA to knock down CBF-B. Under such conditions, GATA4 protein expression was also downregulated (Figure 4C). These results suggest that CBF/NF-Y binding activity toward CCAAT box within the \textit{Gata4} promoter is activated by CH in RV.

Regulation by Annexin A1 Carbonylation
To understand the mechanism of CBF/NF-Y activation, which might promote \textit{Gata4} gene transcription, we searched for proteins that interact with CBF/NF-Y. Immunoprecipitation-immunoblotting revealed that annexin A1 can interact with CBF/NF-Y. Immunoprecipitation of rat RV nuclear extracts with CBF-B antibody, followed by immunoblotting with annexin A1 antibody, showed a strong band, whereas control experiments with normal IgG or in the absence of
nuclear extracts did not (Figure 5A). Similar results were
obtained when immunoprecipitation with annexin A1 anti-
body was followed by blotting with CBF-B antibody (Figure
S4A). This interaction of annexin A1 is inhibitory to CBF/
NF-Y for its DNA binding activity toward CCAAT box, as
the inclusion of recombinant annexin A1 in binding reaction
mixtures for EMSA significantly inhibited probe 2 binding
activity (Figure 5B). Thus, CBF/NF-Y can bind to annexin
A1, which serves as a negative regulator for the DNA binding
activity. We also found that CH decreased the level of
annexin A1 in RV, but not in LV (Figure 5C), suggesting a
mechanism for CBF/NF-Y activation by hypoxic PH in RV,
which mediates the degradation of a negative regulator,
annexin A1. Time-course studies revealed that decrease in
nuclear extracts did not (Figure 5A). Similar results were
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which mediates the degradation of a negative regulator,
annexin A1. Time-course studies revealed that decrease in

Figure 3. Role of the CCAAT Box. A, Sequences of various double-stranded EMSA probes. B, Nuclear extracts from rat RV (7-day
hypoxia-treated), mouse heart or HL-1 cardiac muscle cells were subjected to EMSA using wild-type or mutant probe 2. C, Nuclear
extracts were subjected to EMSA with wild-type or truncated probe 2. D, Heart nuclear extracts were subjected to EMSA using
32P-labeled probe 2 in the presence of excess cold competitor (unlabeled probe 2). E, HL-1 cells were transfected with the luciferase
construct controlled by the 250-bp proximal region of the wild-type (WT) or mutant (Mut) Gata4 promoter. Values represent
means±SEM of the ratio of 250-bp Gata4 promoter-controlled firefly luciferase activity to thymidine kinase (TK) promoter-controlled
Renilla luciferase activity (n=6). The asterisk indicates significant difference from the wild-type Gata4 promoter. Some images were

grouped from different parts of the same gel, and such arrangements are indicated by dividing lines.

Figure 4. Identification of CCAAT box binding protein. A, EMSA was performed with probe 2 and nuclear extracts from rat RV (7-day
hypoxia-treated) or HL-1 cells. Supershift experiments were performed with antibodies (ab) against C/EBPβ and CBF-B. B, Nuclear
extracts from rat RV (7-day hypoxia-treated) or HL-1 cells were mixed with biotinylated probe 2 and streptavidin-agarose. Samples
were centrifuged, washed, and boiled. Supernatant of the final spin was subjected to SDS-PAGE and immunoblotted with CBF-B anti-
body. Lanes denoting minus pull down show control experiments without biotinylated probe 2. WB indicates Western blot. C, HL-1
cells were transfected with small interfering RNA (siRNA) for CBF-B, and nuclear extracts were prepared and subjected to Western
blotting. Values in bar graphs represent means±SEM (n=6). Cont indicates control. Asterisks indicate significant difference.

Wild type  | CTTAGGCCGGCCCTACGGGGGGG
Mutant    | CTATTAAAGCCCTACGGGGGGG
Truncated | CAGGGGATCGTACCCGGGGGGG

C/EBPβ ab | rat1  rat2  rat1  rat2
CBF-B ab   | +    +    -    -

WB: CBF-B

siRNA cont | CBF-B GATA4 Actin

GATA4/Actin (% control)  cont CBF-B siRNA

100 0

CBF-B/Actin (% control) cont CBF-B siRNA

100 0

*
annexin A1 protein expression occurs at 6 hours without changes in CBF-B protein expression (Figures 5C, S4B, and S4C). While no differences in levels of protein expression of annexin A1 in normal RV and LV were noted (Figure 5D), we found a significantly higher level of CBF-NF-Y protein expression in normal RV compared to LV (Figure 5E).

Our laboratory previously found that annexin A1 undergoes proteasome-dependent degradation via protein carbonylation signaling in smooth muscle cells. Similarly, in RV, hypoxia promoted carbonylation of annexin A1 (Figure 6A). These results suggest a possible mechanism for CBF/NF-Y activation, which involves annexin A1 carbonylation and subsequent degradation, suppressing the inhibitory activity of annexin A1 toward CBF/NF-Y. To directly test this hypothesis, effects of deferoxamine (an iron chelator that inhibits iron-catalyzed protein carbonylation) and MG132 (a proteasome inhibitor) were evaluated. Administration of deferoxamine caused inhibition of hypoxic PH-mediated increase in annexin A1 carbonylation (Figure S5) and Gata4 mRNA expression in RV (Figure 6B) as well as RV hypertrophy (Figure 6C). Neither hypoxia nor deferoxamine influenced the expression of other genes such as Gapdh (Figure S6A), suggesting that these events do not occur nonspecifically. Deferoxamine injection to normoxic control animals did not influence the Gata4 mRNA expression (Figure S6B), RV mass (Figure S6C), or RV systolic pressure (22.7±1.0 mm Hg untreated versus 22.4±0.5 mm Hg deferoxamine treated). Similarly, MG132 injection into rats inhibited hypoxic PH-induced increase in Gata4 mRNA expression in the RV (Figure S7). MG132 injection to normoxic control animals did not influence the Gata4 mRNA expression (Figure S7) or RV systolic pressure (22.7±1.0 mm Hg untreated versus 23.3±1.6 mm Hg MG132 treated).

**Discussion**

Right heart failure is the major cause of death among PH patients; however, pathophysiology of the right heart is not well understood. Since agents to treat left heart failure do not necessarily work to treat right heart failure, understanding of the right heart biology is needed to develop therapeutic strategies to specifically prevent/treat right heart failure in PH patients. The present work investigated regulatory mechanisms of GATA4 in RV of rats subjected to CH to induce PH. We found a novel mechanism of GATA4 activation that involves the promotion of Gata4 gene transcription that is regulated by signaling via metal-catalyzed oxidation. This may define the difference between hypertrophic signaling mechanisms of RV and LV.

GATA4 is a major transcriptional regulator of cardiac hypertrophy. GATA4 activation mechanisms are well understood in the context of LV hypertrophy and involve posttranslational modifications. In the LV of rats subjected to pressure overload by aortic constriction, GATA4 activity was increased without changes in GATA4 expression. Thus, activation mechanisms of GATA4 in LV seem to involve posttranslational modifications. In contrast, in RV, increased mRNA expression of Gata4 was noted in rats subjected to PH either by pulmonary artery banding or by CH as described in this study. These results suggest that activation mechanisms of GATA4 in response to pressure overload are different between LV and RV.

Since understanding RV-specific mechanisms responding to pressure overload has great clinical significance for finding treatment/management strategies for PH patients, the present study examined the detailed mechanism of increased Gata4 gene transcription in RV. We cloned the Gata4 promoter and identified a major regulatory element, CCAAT box. This element is necessary for the basal expression of GATA4, and
The binding toward this element is activated in response to hypoxic PH in RV. The regulatory mechanism of \textit{Gata4} gene transcription involves CBF/NF-Y, which exerts differential regulation on a wide variety of genes through its interaction with CCAAT box.\textsuperscript{18} As roles of CBF/NF-Y in the heart have not been studied, the finding in this study that pressure overload activates this transcription factor is novel and may have revealed an important biological mechanism in the heart.

To understand the activation mechanism, we screened for proteins that can interact with CBF/NF-Y. We found that annexin A1 interacts with CBF/NF-Y. The addition of recombinant annexin A1 in the reaction mixture for EMSA inhibited the binding of CBF/NF-Y to CCAAT box, revealing that annexin A1 is a negative regulator of CBF/NF-Y. Our laboratory previously reported a novel oxidant signaling mechanism involving protein carbonylation and subsequent proteasome-dependent degradation of annexin A1.\textsuperscript{17} In RV of rats subjected to hypoxic PH, annexin A1 is carbonylated, and its expression is reduced. The reduced annexin A1 expression should liberate CBF/NF-Y, promoting its DNA binding activity and thus \textit{Gata4} gene transcription.

Protein carbonylation often occurs in response to metal-catalyzed oxidation. The role of this mechanism in PH-mediated increase in \textit{Gata4} gene transcription as well as RV hypertrophy is supported by our observations that an iron chelator, deferoxamine, effectively inhibited hypoxic PH-mediated increase in \textit{Gata4} mRNA expression and RV hypertrophy. Since deferoxamine does not inhibit hypoxic pulmonary vasoconstriction,\textsuperscript{19} deferoxamine likely directly influences the RV hypertrophic mechanism. These results also suggest the possible use of deferoxamine or other inhibitors of metal-catalyzed oxidation to inhibit the development of RV hypertrophy in PH patients.

We propose 2 possible mechanisms by which the signaling pathway described in this study may preferentially occur in RV compared to LV. We found that the expression of CBF/NF-Y is higher in RV compared to LV. This may increase the sensitivity of RV to activate CBF/NF-Y by being liberated from annexin A1 as this protein gets degraded. In the previous study,\textsuperscript{20} we found that protein carbonylation mediated by serotonin is more pronounced in RV than in LV, indicating that the sensitivity to carbonylation signaling might be higher in RV. We attributed this to be due to differential expression of monoamine oxidase between RV and LV. Thus, this is another possible mechanism in which

\begin{figure}
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\caption{Role of metal-catalyzed protein carbonylation in \textit{Gata4} gene expression and the development of RV hypertrophy. A, Rats were subjected to hypoxia, and RV and LV homogenates were prepared. Carbonylated annexin A1 was monitored by labeling carbonylated proteins with 2,4-dinitrophenylhydrazine (DNPH), immunoprecipitated with the antibody for DNPH-derivatized proteins, and Western blotting with annexin A1 antibody. Values in bar graphs represent means \pm SEM of carbonylated annexin A1 and the ratio of carbonylated annexin A1 to annexin A1 protein level (n=6). Asterisks indicate significant difference from control. B, Rats were given injections of deferoxamine (DFO; 20 mg/kg) or saline daily during the 4-day exposure to hypoxia. \textit{Gata4} mRNA and 28S rRNA levels were monitored by reverse transcription-polymerase chain reaction. Values in the bar graph represent means \pm SEM (n=5). C, Masses of RV, LV, and septum (S) were measured, and RV/(LV+S) values were calculated as an estimate of RV hypertrophy (n=3). Asterisks indicate significant difference from each other.}
\end{figure}
the activation of Gata4 gene transcription in response to pressure overload preferentially occurs in RV.

There are limitations in this study. First, while the previous study has shown that pulmonary arterial banding increases Gata4 gene transcription, the present study using the CH model does not distinguish between direct effects of pressure overload and possible influence by hypoxia on specific pathways we propose. However, mechanisms described in this study are not merely hypoxia-responsive cardiac signaling, because hypoxia did not trigger these events in LV. Second, in our studies of intact animals, we observed the activation of sequential signaling events, which comprise annexin A1 carbonylation/degradation, CBF/NF-Y activation, and Gata4 gene transcription. These observed events in vivo, which occur slower than what might be expected in cell signaling from studies of cultured cells, may offer invaluable pathophysiologically relevant information.

**Perspectives**

The present study, for the first time, demonstrates an RV-specific signaling mechanism in response to pressure overload. This pathway involves metal-catalyzed carbonylation and subsequent degradation of annexin A1, which liberates CBF/NF-Y for the activation of Gata4 gene transcription (Figure S8). We propose that this mechanism may influence clinically important events of RV hypertrophy in PH patients. Further understanding of these mechanisms may lead to the development of therapeutic strategies to reduce morbidity and mortality from right heart failure.

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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% O₂ with influx of N₂. Ventilation was adjusted to remove CO₂, so that its level does not exceed 5,000 ppm. Normoxia controls were subjected to ambient 21% O₂ in another chamber. Animals were fed normal rat chow 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. 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) ³²P-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 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.

Western blot analysis

Samples were prepared as previously described. 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.
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\textsuperscript{14} 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\textsuperscript{9} 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 AAC GAC GTG 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 GGC AT–3’; CCAAT mut-R, 5’-CCT CGC 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


Park et al. Fig. S1

A

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C

D

E

Day 0  Day 2  Day 7  Day 14
Park et al. Fig. S1

**F**

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**G**

Myocyte width (µm) vs. Hypoxia (days)

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Fig. S1: Hypoxic pulmonary hypertension induces right ventricular (RV) hypertrophy. Rats were subjected to chronic hypoxia at 10% O₂ 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) Verhoeff’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 glyceraldehyde 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.
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\textsubscript{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.
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).
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% O₂ 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% O₂. 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).
**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% O₂ 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.
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.