NFATc3 contributes to intermittent hypoxia-induced arterial remodeling in mice

Sergio de Frutos,1 Elizabeth Caldwell,1 Carlos H. Nitta,1 Nancy L. Kanagy,1 Jian Wang,2 Wei Wang,2 Mary K. Walker,3 and Laura V. Gonzalez Bosc1

1Vascular Physiology Group, Department of Cell Biology and Physiology, School of Medicine, 2Department of Chemistry and Biological Chemistry, and 3College of Pharmacy, University of New Mexico, Albuquerque, New Mexico

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SLEEP APNEA (SA) is defined as intermittent respiratory arrest during sleep and affects up to 20% of the adult population. One of the major consequences is decreased oxygen saturation (hypoxia) and increased arterial content of CO2 (hypercapnia). SA appears to directly initiate vascular changes that predispose individuals to cardiovascular disease, and SA patients have increased incidence of hypertension, peripheral vascular disease. Exposing rodents to intermittent hypoxia during sleep mimics the cyclical hypoxia/normoxia of SA. We have previously shown that in mice and rats intermittent hypoxia induces ET-1 upregulation and systemic hypertension. Furthermore, intermittent hypoxia (IH) in mice increases nuclear factor of activated T cells isoform 3 (NFATc3) transcriptional activity in aorta and mesenteric arteries, whereas the calcineurin/NFAT inhibitor cyclosporin A prevents IH-induced hypertension. More importantly, NFATc3 knockout (KO) mice do not develop IH-induced hypertension. The goals of this study were to determine the role of NFATc3 in IH-induced arterial remodeling and whether IH-induced NFATc3 activation is mediated by ET-1. Oral administration of both a dual (bosentan) and a selective endothelin receptor type A antagonist (PD155080) during 2 days of IH exposure attenuated NFAT activation in aorta and mesenteric arteries, Rho kinase inhibition with fasudil also prevented IH-induced NFAT activation. Mesenteric artery cross-sectional wall thickness was increased by IH in wild-type (WT) and vehicle-treated mice but not in bosentan-treated and NFATc3 KO mice. The arterial remodeling in mesenteric arteries after IH was characterized by increased expression of the hypertrophic NFATc3 target smooth muscle-α-actin in WT but not in KO mice. These results indicate that ET-1 is an upstream activator of NFATc3 during intermittent hypoxia, contributing to the resultant hypertension and increased wall thickness.

arteries; hypertension; endothelin 1; Rho kinase

Sleep apnea (SA) is defined as intermittent respiratory arrest during sleep and affects up to 20% of the adult population. One of the major consequences is decreased oxygen saturation (hypoxia) and increased arterial content of CO2 (hypercapnia). SA appears to directly initiate vascular changes that predispose individuals to cardiovascular disease, and SA patients have increased incidence of hypertension, peripheral vascular disease, stroke and sudden cardiac death [reviewed in (9, 27)].

Our group has recently demonstrated that rats and mice exposed to intermittent hypoxia (IH) with CO2 supplementation during sleep have elevated blood pressure (6, 18), elevated circulating ET-1 (18), upregulation of pre-pro ET-1 in lungs (6), and blood pressure is normalized with ET antagonists (1, 18). These studies, together with clinical studies showing increased circulating ET-1 in SA (5, 37), suggest that augmented ET-1 vasoconstriction contributes to SA hypertension. However, there is no direct information about the role of ET-1 in IH-induced hypertension and vascular remodeling in mice.

We have recently demonstrated that the transcription factor nuclear factor of activated T cells isoform 3 (NFATc3) is activated in aorta and mesenteric arteries (MA) from mice exposed to IH and is required for IH-induced systemic hypertension (6).

NFATc3 belongs to the Rel-family of transcription factors, which includes NF-κB. In smooth muscle, NFATc3 nuclear accumulation is increased by Gq-coupled receptor agonists such as UTP, ANG II, and ET-1. This accumulation is mediated by the phosphatase calcineurin and is dependent on both endoplasmic reticulum Ca2+ release through inositol trisphosphate receptors (IP3R) and extracellular Ca2+ influx through voltage-dependent Ca2+ channels (11, 12, 14, 29).

RhoA/Rho kinase (ROK) also appears to activate NFAT. In rat smooth muscle, the ROK inhibitor Y-27632 significantly attenuates agonist-induced NFAT nuclear localization (43). ROK is a ubiquitously expressed serine-threonine protein kinase activated by RhoA, a small monomeric GTPase that becomes active after exchange of bound GDP for GTP with subsequent membrane translocation (19). RhoA and ROK can be activated by many agonists, including ET-1 (26, 38). Although several agonists that activate ROK have been implicated in NFATc3 activation, the role of RhoA/ROK in NFATc3 activation in mouse systemic arteries has not been addressed.

NFATc3 is involved in vascular smooth muscle differentiation (13), contractility (2, 28), and hypertrophy (7). In mice exposed to chronic hypoxia (CH), NFATc3 upregulates the hypertrophic marker smooth muscle (SM)-α-actin (13) in pulmonary arterial smooth muscle and is required for CH-induced increases in pulmonary arterial wall thickness (7).

These intriguing observations together with reports showing that patients with sleep apnea have increased markers of vascular remodeling (40) suggest NFATc3 could contribute to systemic vascular SM hypertrophy. Therefore, we hypothesized that IH increases ET-1 activation of NFATc3 to increase SM-α-actin expression and arterial wall thickness, markers of arterial remodeling.

In support of the hypothesis, our data demonstrate that with IH exposure, ET-1 acting on endothelin receptor type A (ETaR), activates NFAT in aorta and MA, and ROK contributes to this response. Furthermore, IH-induced SM-α-actin upregulation and increased arterial wall thickness requires NFATc3 and is mediated by ET-1 receptor activation.

Address for reprint requests and other correspondence: L. V. Gonzalez Bosc, Cell Biology and Physiology, School of Medicine, Univ. of New Mexico, MSC08 4750, Albuquerque, NM 87131 (e-mail: iongalezbosc@salud.unm.edu).
METHODS

All protocols employed in this study were reviewed and approved by the Institutional Animal Care and Use Committee of the University of New Mexico, Health Science Center (Albuquerque, NM).

Animals. Adult male 9x-NFAT-luciferase reporter (NFAT-luc, FVBN background), NFATc3 knockout (NFATc3 KO, Balb/C background), and Balb/C wild-type (WT) mice (25–30 g) were used. NFAT-luc mice were provided by Dr. Jeffery D. Molkentin (Children’s Hospital Medical Center, Cincinnati, OH) (4, 28). NFATc3 KO mice were kindly provided by Dr. Laurie Glimcher (Harvard University) (45).

Treatments and intermittent hypercarbic/hypoxia exposure. PD155080 (50 µg·kg⁻¹·day⁻¹), selective ET₄R antagonist, or placebo was administered in oral dough pellets for 3 days (College of Pharmacy, University of New Mexico) (45). Bosentan (30 mg·kg⁻¹·day⁻¹), a dual ET receptor antagonist, was administered in the drinking water for 3 or 7 days. Bosentan was kindly provided by Actelion Pharmaceuticals (Switzerland). Fasudil (30 mg·kg⁻¹·day⁻¹; LC Laboratories), a Rho kinase antagonist, was administered subcutaneously via osmotic pumps (Alzet) for 3 days.

The animals were housed in regular cages with snug-fitting Plexiglas lids. During the normal sleep period, air in the cage was cycled between a low-oxygen (5% O₂), high carbon dioxide (5% CO₂) environment and room air, experiencing the same 12-h:12-h light-dark cycle. The mice were acclimated for 5 days before recording blood pressure. The CODA system utilizes volume pressure recording (VPR) technology to measure mouse blood pressure parameters: systolic blood pressure, diastolic blood pressure, heart rate, mean blood pressure, tail blood flow, and tail blood volume. VPR is clinically validated and provides close to 100% correlation with telemetry and direct blood pressure measurements for systolic and diastolic blood pressure (24, 33).

Immunofluorescence confocal microscopy. Isolated MA (second, third, and fourth order; outer diameter 100 to 500 µm) were dissected from the surrounding connective tissue. Lungs were stored in RNAlater (Ambion). Total RNA was isolated using the RNeasy Mini Kit (Qiagen). Total RNA was reverse transcribed to cDNA using a high capacity reverse transcription kit (A&B). For real-time detection of pre-pro ET-1 transcripts, SYBR Green Master Mix (A&B) was used as previously described (6). The normalized gene expression method (2⁻ΔΔCT) for relative quantification of gene expression was used (23).

Luciferase activity. Isolated arteries from NFAT-luc mice were lysed (Promega buffer). Luciferase activity was measured using a Luciferase Assay System kit (Promega), and light was detected with a luminometer (TD20/20; Turner). Protein content determined by the Bradford method (Bio-Rad) was used to normalize luciferase activity.

Quantitative RT-PCR. Isolated arteries and lungs were stored in RNA later (Ambion). Total RNA was isolated using the RNeasy Mini Kit (Qiagen). Total RNA was reverse transcribed to cDNA using a high capacity reverse transcription kit (A&B). For real-time detection of SM-α-actin transcripts (Mm01546133_mi) and reference gene (18S, 4319413E-0502018), TaqMan Gene Expression Assays (A&B) were used. For real-time detection of pre-pro ET-1 transcripts, SYBR Green Master Mix (A&B) was used as previously described (6). The normalized gene expression method (2⁻ΔΔCT) for relative quantification of gene expression was used (23).

Blood pressure was monitored using the CODA 2 Noninvasive Blood Pressure System (Kent Scientific, Torrington, CT) immediately after the daily cycling. Animals were acclimated for 5 days before recording blood pressure. The CODA system utilizes volume pressure recording (VPR) technology to measure mouse blood pressure parameters: systolic blood pressure, diastolic blood pressure, heart rate, mean blood pressure, tail blood flow, and tail blood volume. VPR is clinically validated and provides close to 100% correlation with telemetry and direct blood pressure measurements for systolic and diastolic blood pressure (24, 33).

**Fig. 1.** Both a dual and a selective endothelin receptor type A antagonist prevent intermittent hypoxia (IH)-induced nuclear factor of activated T cells (NFAT) activation in aorta and mesenteric arteries (MA). Luciferase activity was measured in aorta and MA from NFAT-luc animals treated with bosentan (30 mg·kg⁻¹·day⁻¹ in drinking water; A) and PD155080 (PD; 50 µg·kg⁻¹·day⁻¹ in oral pellets; B) for 3 days and exposed to IH during the last 2 days of treatment. RLU, relative luciferase units. *P < 0.01 vs. IH vehicle; #P < 0.05 vs. IH vehicle; 2-way ANOVA and Bonferroni’s post test; n = 5–7 animals.
was confirmed by the absence of fluorescence in tissues incubated with primary or secondary antibodies alone. For scoring of SM cells (SMC) NFATc3-positive nuclei, multiple fields for each vessel were imaged and counted by two independent observers using Metamorph software (Universal Imaging). The software was programmed so that individual pixels appear white instead of yellow if the green nuclear acid stain and red NFATc3 stain colocalized. Thus a cell was considered positive if colocalization (white) was uniformly distributed in the nucleus and negative if no colocalization (green only) was observed (6, 7, 11, 14).

Vascular morphometry. Animals were anesthetized with 5% isoflurane in O2 and perfused via the left ventricle with 20 ml of modified physiological saline solution HEPES-PSS (in mM) 134 NaCl, 6 KCl, 1 MgCl, 10 HEPES, 0.026 EDTA, 5 EGTA, and 10 glucose containing heparin, 4% albumin (Sigma), and 10 4M papaverine (Sigma), at a rate close to normal cardiac output in mice (17 ml/min) to maximally dilate and flush the circulation of blood. Mice were then perfused with 4% formaldehyde (Polyscience) and 10 4M papaverine (Sigma) in PBS at the same rate (7). Following fixation, the aorta and MA were embedded in paraffin. Sections (5 µm) were stained with hematoxylin-eosin and examined with a 40× or 20× objective on a Nikon Diaphot 300 microscope and imaged with a cooled digital charge-coupled device (CCD) camera (Photometrics SenSys 1400). Images were processed with ImageJ (National Institutes of Health). Vessels sectioned at oblique angles were excluded from analysis. To determine cross-sectional wall thickness, the perimeters of the internal and external elastic laminae were traced and the distance between was calculated (7).

To confirm the morphometric studies, MA (third order) were isolated and dissected from the surrounding connective tissue. Arteries were then cannulated, pressurized to 60 mmHg, and mounted in a specially designed, close working-distance arteriograph. In arteries fully dilated in a Ca2+-free EGTA (5 mM)-containing PSS inner and outer diameters were recorded with an edge detection system (Ion Optix), and wall thickness was calculated as a percentage of outer diameter.

Cell proliferation assay. Animals were injected with 0.1 ml 5-ethynyl-2'-deoxyuridine (EdU; 1 mg/m) in saline each of the last 2 days before euthanasia. MA (second, third, and fourth order) were isolated and MA were embedded in paraffin. Sections (5 µm) were stained with hematoxylin-eosin and examined with a 40× or 20× objective on a Nikon Diaphot 300 microscope and imaged with a cooled digital charge-coupled device (CCD) camera (Photometrics SenSys 1400). Images were processed with ImageJ (National Institutes of Health). Vessels sectioned at oblique angles were excluded from analysis. To determine cross-sectional wall thickness, the perimeters of the internal and external elastic laminae were traced and the distance between was calculated (7).

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and fixed with 4% formaldehyde in PBS. Whole arteries were stained with Click-iT EdU Assay from Invitrogen to detect proliferating endothelial cells (EC), SMC, and adventitia cells (AC) by fluorescence microscopy. Nuclei were counterstained with Hoechst 33342.

RESULTS

IH-induced NFATc3 activation is mediated by ETAR. We previously demonstrated that NFATc3 is the NFAT isoform activated by IH in aorta and MA (6), supporting our focus on this isoform. In addition, pre-pro ET-1 mRNA is increased in lungs of IH exposed mice compared with air controls (6) and correlates with the degree of NFAT activation in aorta and MA, suggesting ET-1 mediates IH-induced NFAT activation. Lung tissue is a very important source of circulating ET-1 (8).

To determine whether ET receptors mediate IH-induced NFAT activation, NFAT-luc mice received bosentan, a dual ET receptor antagonist, before and during IH exposure. Bosentan-treated mice did not have increased NFAT activity, whereas vehicle-treated mice had increased activity in both aorta and MA (Fig. 1A). These data indicate ET-1 mediates NFAT induction by IH.

To determine whether ETAR mediates IH-induced NFAT activation, NFAT-luc mice received placebo or the ETAR antagonist PD155080 before and during 2 days of IH treatment. As with bosentan, the increase in NFAT activity observed in vehicle mice exposed to IH was abolished by PD155080 (Fig. 1B), suggesting ET-1 acting through ETAR mediates IH-induced activation of NFAT. No effect of PD155080 or bosentan was observed in arteries from air mice.

ROK activity is required for IH-induced NFATc3 activation. RhoA/ROK pathway is essential for NFAT activation in B cells (10, 15) and mediates NFAT activation in rat pulmonary arteries. Furthermore, ET-1 can activate ROK, suggesting ET-1-mediated IH-induced NFAT activation may require ROK. We observed that treating NFAT-luc mice with the ROK inhibitor fasudil before and during IH treatment completely prevented IH-induced increases in NFAT reporter activity both in aorta and MA (Fig. 2). Therefore, ROK is required for...
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**ROK activity is required for ET-1-induced NFATc3 activation** *ex vivo*. To demonstrate that the effect of fasudil in vivo was a direct effect on arterial cells, isolated MA were incubated with ET-1 in the presence and absence of fasudil. NFATc3 nuclear accumulation in SMC was then determined. As expected, ET-1 induced a significant increase in NFATc3 nuclear accumulation and fasudil prevented that increase (Fig. 3, A and B).

**NFATc3 is required for IH-induced arterial remodeling.** We have previously shown that NFATc3 is required for pulmonary arterial remodeling in CH-induced pulmonary hypertension (7). In addition, our group and others have shown that NFATc3 is required for the development of systemic hypertension induced by IH and ANG II, respectively (6, 28). To determine whether NFATc3 is also required for arterial remodeling in our model, vascular morphology studies were performed in aorta and MA (100 to <500 μm outer perimeter) in NFATc3 WT and KO exposed to air or IH for 1 wk.

We have previously shown that 7 days of IH significantly increases mean arterial pressure (MAP) in NFATc3 WT but not in NFATc3 KO mice (ΔMAP = 19 ± 4 vs. −9 ± 8; P < 0.05; n = 5) (6).

Figure 4, A and B, shows that MA cross-sectional wall thickness is increased in WT mice exposed to 7 days of IH compared with air controls. However, that increase is absent in MA from KO mice. No changes in aorta wall thickness were

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**Fig. 5. Dual ET antagonist prevents IH-induced mesenteric arterial remodeling.** A: mean arterial pressure (MAP) was measured in mice treated with vehicle or bosentan (30 mg·kg⁻¹·day⁻¹) exposed to air or IH for 7 days. *P < 0.05 vs. day 0; #P < 0.05 vs. air vehicle, 2-way ANOVA and Bonferroni’s post test; n = 6 animals.

B: arterial wall thickness was measured in hematoxylin-eosin-stained sections of mesenteric arteries from mice treated with vehicle or bosentan (30 mg·kg⁻¹·day⁻¹) exposed to air or IH for 7 days. Representative images are shown. Scale bar = 50 μm. C: summary data. *P < 0.01 vs. all groups; 2-way ANOVA and Bonferroni’s post test; n = 6 animals.
observed (data not shown). To validate the histology studies, wall thickness was determined in cannulated fully dilated MA from air and IH NFATc3 WT and KO mice. Consistently with the histological studies, MA from NFATc3 WT mice exposed to 7 days of IH have a significant increase in wall thickness compared with the air group (Fig. 4C). MA from IH exposed NFATc3 KO mice do not show any significant change in wall thickness (Fig. 4C). These results demonstrate that NFATc3 is required for IH-induced resistance artery remodeling.

To determine whether the difference between the response of NFATc3 WT and KO to IH is due to differences in ET-1, pre-pro ET-1 mRNA content were measured in lung from NFATc3 WT and KO exposed to air or 7 days of IH, as previously described (6). IH caused a similar increase in pre-pro ET-1 mRNA in both NFATc3 WT and KO mice (2ΔΔCT, air WT = 1.01 ± 0.07; air KO = 1.07 ± 0.18; IH 7 days WT = 2.80 ± 0.58*; IH 7 days KO = 3.42 ± 0.47*; *P < 0.05 vs. air WT and air KO; n = 5). Knockdown of NFATc3 did not alter pre-pro ET-1 mRNA content in air- or IH-exposed mice. These results suggest the ET-1 system is intact in the NFATc3 KO mice, but the response to ET-1 is impaired.

Bosentan prevents IH-induced arterial remodeling and hypertension. We previously demonstrated that NFATc3 is required for IH-induced hypertension (6), and our data suggest ET-1 is an upstream activator of NFATc3 in MA during IH (Fig. 1A). Consistently, we observed that ET receptor inhibition with bosentan prevents both IH-induced increases in MAP (Fig. 5A) and arterial wall thickness (Fig. 5, B and C).

NFATc3-mediated IH-induced arterial remodeling does not involve vascular cell proliferation. Previous studies have demonstrated a role for NFAT in cell proliferation (3, 22, 30, 44). To determine whether NFATc3-mediated IH-induced MA remodeling is due to increased proliferation of EC, SMC, and/or AC, 2- and 7-day IH- and air-exposed NFATc3 WT and KO mice were injected with EdU for 2 days before euthanasia. We observed no significant changes in the number of proliferating SMC and AC in any of the groups (Fig. 6, B and C). However, the number of EC was significantly lower in MA from 7 days IH-exposed NFATc3 WT and KO mice (Fig. 6A), suggesting that NFATc3 is not involved in this IH-induced reduction in EC proliferation.

NFATc3 is required for IH-induced SM-α-actin upregulation. Since we have previously shown that NFATc3 transactivates SM-α-actin promoter and upregulates its expression in arterial myocytes (7, 13), changes in α-actin mRNA levels were
measured in MA from WT and KO mice exposed to air or IH for 2 and 7 days.

SM-α-actin mRNA significantly increased in NFATc3 WT mice exposed to IH for 2 and 7 days. The increase was absent in NFATc3 KO mice (Fig. 7). These results suggest that NFATc3 is required for IH-induced MA remodeling accompanied by an increase in the hypertrophic and differentiation marker SM-α-actin.

**DISCUSSION**

This study demonstrates that ET-1, acting through ETAR, is an important upstream factor in IH-induced NFATc3 activation in the vasculature, potentially through ROK activation. In addition, NFATc3 is required for IH-induced increases in MA wall thickness including increased SM-α-actin expression, strongly suggestive of smooth muscle hypertrophy.

Our findings are consistent with the previously demonstrated role for ET-1 as a mediator of hypertension in both SA animal models (1, 18) and patients (5, 37). Furthermore, our data demonstrate that ET-1 is an upstream activator of NFAT since both ET-1 antagonists prevented NFAT activation. However, these studies do not exclude contributions by other factors such as ANG II, which has been implicated in the development of hypertension in SA patients and in animal models of SA (5, 27, 35, 37). Of interest, several studies have shown cross talk between ET-1 and ANG II. In particular, administration of an angiotensin converting enzyme inhibitor reduces elevated plasma concentrations of ET-1 in hypertensive mice (25). In addition, ANG II increases ET-1 synthesis via elevated reactive oxygen species (ROS) in human arteries in culture, and there is a significant association between elevated ANG II levels, increased oxidative stress, and increased ET-1 concentrations in patients with atherosclerosis (20). Thus, the potential role of ANG II and ROS in ET-1-mediated NFAT activation in IH remains to be explored.

Although it has been previously shown that ROK is required for NFAT activation in rat pulmonary arteries (43) and immune cells (10, 15, 42), to our knowledge this is the first report demonstrating that ROK activity is required for NFAT activation in mouse aorta and MA. The mechanism by which RhoA/ROK activates NFAT is unclear, but evidence indicates it may involve actin polymerization (42). Beside the recognized role of ROK in contraction, it is also involved in actin cytoskeleton organization, cell adhesion and motility, regulation of gene expression, and hypertrophy through induction of the early growth response genes c-fos and c-jun (AP-1) (16, 17, 34). Because AP-1 is a cofactor of NFAT (36), ROK-mediated induction of AP-1 may be enhancing NFAT transcriptional activity.

The observed increase in wall thickness of small arteries in WT mice exposed to IH is consistent with a previous report of upregulated vascular hypertrophy markers SM myosin and protein-disulfide isomerase in the kidneys of rats exposed to IH (41). IH also has been shown to increase intimal thickening after arterial balloon injury in rabbits (21). Finally, patients with obstructive SA and coronary artery disease have a higher degree of late lumen loss, a marker of restenosis and vessel remodeling, after elective percutaneous intervention (40), suggesting SA leads to vascular remodeling and hypertrophy in both animals and humans.

The absence of MA remodeling in NFATc3 KO could be a consequence of the absence of IH-induced hypertension in these animals (6) or a decrease in IH-induced ET-1 upregulation. However, IH increases ET-1 in both NFATc3 WT and KO mice, and ex vivo treatment of arteries with ET-1 increases NFATc3 nuclear translocation in the absence of intravascular pressure. These findings together with previous reports suggest that IH causes ET-1 upregulation. ET-1, by acting on ETAR, then increases intracellular Ca\(^{2+}\) and ROK to activate the calcineurin/NFATc3 pathway. NFATc3 in turn upregulates the expression of genes responsible for the hypertension and SM hypertrophy. In support of this conclusion, bosentan prevented NFAT activation, IH-induced hypertension, and increased arterial wall thickness.

Increased arterial wall thickness or remodeling can be caused by increased SMC and/or AC proliferation and/or hypertrophy of SMC. Our results suggest that IH does not induce either SMC or AC proliferation. On the contrary, our results suggest that NFATc3 activation induces upregulation of SM-α-actin expression and increases arterial wall thickness. The upregulation of SM-α-actin suggests the increased arterial wall thickness is due to SM hypertrophy since, in adults, SM-α-actin expression generally occurs only in cells of myogenic lineage during hypertrophy (32).

In summary, IH increases ET-1 and induces NFAT activation through ETAR and ROK. This induction of NFATc3 is required for IH-induced increases in wall thickness and SM-α-actin mRNA upregulation. Therefore, NFATc3 activation may contribute to SA-induced vascular remodeling and hypertension, and these studies define a novel regulatory pathway of gene transcription activated during intermittent hypoxia that could be therapeutically targeted.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

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