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NFATc3 is required for intermittent hypoxia-induced hypertension

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de Frutos S, Duling L, Alò D, Berry T, Jackson-Weaver O, Walker M, Kanagy N, González Bosc L. NFATc3 is required for intermittent hypoxia-induced hypertension. *Am J Physiol Heart Circ Physiol* 294: H2382–H2390, 2008. First published March 21, 2008; doi:10.1152/ajpheart.00132.2008.—Sleep apnea, defined as intermittent respiratory arrest during sleep, is associated with increased incidence of hypertension and peripheral vascular disease. Exposure of rodents to brief periods of intermittent hypercarbia/hypoxia (H-IH) during sleep mimics the cyclical hypoxia-normoxia of sleep apnea. Endothelin-1, an upstream activator of nuclear factor of activated T cells (NFAT), is increased during H-IH. Therefore, we hypothesized that NFATc3 is activated by H-IH and is required for H-IH-induced hypertension. Consistent with this hypothesis, we found that H-IH (20 brief exposures per hour to 5% O_2-5% CO_2 for 7 h/day) induces systemic hypertension in mice [mean arterial pressure (MAP) = 97 ± 2 vs. 124 ± 2 mmHg, $P < 0.05$, $n = 5$] and increases NFATc3 transcriptional activity in aorta and mesenteric arteries. Cyclosporin A, an NFAT inhibitor, and genetic ablation of NFATc3 [NFATc3 knockout (KO)] prevented NFAT activation. More importantly, H-IH-induced hypertension was attenuated in cyclosporin A-treated mice and prevented in NFATc3 KO mice. MAP was significantly elevated in wild-type mice (Δ = 23.5 ± 6.1 mmHg), but not in KO mice (Δ = −3.9 ± 5.7). These results indicate that H-IH-induced increases in MAP require NFATc3 and that NFATc3 may contribute to the vascular changes associated with H-IH-induced hypertension.

nuclear factor of activated T cells; hypercarbic; mouse; endothelin-1; sleep apnea

SLEEP APNEA (SA), defined as intermittent respiratory arrest during sleep, affects up to 20% of the adult population. Among the major consequences of SA are decreased O_2 saturation (hypoxia) and increased CO_2 saturation (hypercapnia). In SA patients, incidence of hypertension, peripheral vascular disease, stroke, and sudden cardiac death is increased (for review see Refs. 21 and 44). Thus SA appears to directly initiate vascular changes that predispose individuals to cardiovascular disease.

Recently, it was demonstrated that exposure of rodents to periods of intermittent hypoxia with CO_2 supplementation [intermittent hypercarbia/hypoxia (H-IH)] during sleep mimics the cyclical hypoxia-normoxia of SA. In rats exposed to H-IH, blood pressure (34, 35) and circulating endothelin-1 (ET-1) are elevated (35), vasoconstrictor sensitivity to ET-1 is increased (2), and blood pressure is normalized with ET antagonists (35). These studies, together with clinical studies showing increased circulating ET-1 in SA (10, 55), suggest that augmented ET-1 vasoconstriction contributes to SA hypertension. The sustained increased blood pressure observed in SA patients and animal models also appears to involve activation of the sympathetic nervous and renin-angiotensin systems and diminished activity of nitric oxide synthase (for review see Refs. 44 and 52).

ET-1 acts through $G\alpha_q$-coupled receptors as a Ca^{2+}-mobilizing agent and as a potent activator of the nuclear factor of activated T cells (NFAT) (1, 25, 57). ANG II, glucose through UTP release, and α_1-adrenergic agonists are also potent stimuli of NFAT activation (1, 25, 27, 46, 57).

NFAT, in turn, serves as a "master transcriptional regulator," interlinking Ca^{2+} signaling with other signaling pathways to induce specific genetic programs. NFAT activation is regulated primarily through control of its subcellular localization (54). Ca^{2+}-elevating stimuli activate the Ca^{2+}-dependent phosphatase calcineurin, which dephosphorylates NFAT, leading to nuclear translocation. NFAT activation is generally associated with an increase in gene expression (31). However, several reports have shown that NFAT may also function as a repressor of gene expression (3, 29, 45).

Of the four members of the NFAT family, NFATc3 is specifically implicated in vascular development (28) and maintenance of the contractile phenotype in vascular smooth muscle cells (VSMC) through regulation of α-actin expression (11, 26, 59). NFATc3 also modulates VSMC contractility by regulating voltage-dependent and large-conductance K^+ channel expression in cerebral arteries (3, 45).

Although many NFAT transcriptional partners are induced by hypoxia [activator protein-1, GATA, and cAMP response element-binding protein (CREB)-binding protein/p300] (4, 34, 38, 53, 61), NFAT activation in SA patients or animal models has not been described.

These intriguing observations led us to hypothesize that H-IH activates NFATc3 in systemic arterial smooth muscle, where it contributes to the development of hypertension.

The aims of the present study were to determine whether *1*) H-IH induces hypertension in mice, *2*) H-IH induces ET-1 upregulation; *3*) H-IH induces NFATc3 activation in systemic arteries; and *4*) NFATc3 is required for H-IH-induced hypertension.

In support of the hypothesis, our data demonstrate that H-IH indeed causes hypertension and increases NFATc3 transcriptional activity in mouse arteries. More importantly, pharmacological inhibition of calcineurin activation of NFAT [cyclosporin (CsA)] or the genetic ablation of NFATc3 prevents H-IH-induced hypertension. In addition, upregulation of ET-1 in the lung is correlated with NFATc3 activation in the aorta. These results suggest that NFATc3 plays a role in the vascular and blood pressure changes observed during H-IH.

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MATERIALS AND METHODS

All protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the University of New Mexico Health Science Center.

Animals. Adult male 9×NFAT-luciferase reporter (NFAT-luc) background strain FVBN, NFATc3-knockout (NFATc3 KO), Balb/C wild-type (WT), and FVBN mice (25–30 g) were used. In addition, NFAT-luc mice were backcrossed with NFATc3 KO mice for at least two generations, and NFAT-luc/NFATc3$^{+/+}$ (WT) and NFAT-luc/NFATc3$^{-/-}$ (KO) mice were used (11). NFAT-luc mice were provided by Dr. Jeffery D. Molkentin (Department of Pediatrics, Children's Hospital Medical Center, Cincinnati, OH) (7), and NFATc3 KO mice were kindly provided by Dr. Laurie Glimcher (Harvard University) (49). Heterozygous mice were bred to obtain age-matched WT (Balb/C) and KO mice. Importantly, it has been shown that loss of NFATc3 does not induce a compensatory upregulation of other NFAT isoforms (60). Cyclosporin A (Calbiochem) or Cremophor EL (vehicle) was administered subcutaneously at 25 mg·kg^{-1}·day^{-1} for 3 or 10 days to FVBN and NFAT-luc mice.

H-IH exposure. 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 (5%) O_2-high (5%) CO_2 environment and room air. In rats, this protocol causes hypoxia but maintains constant plasma CO_2 levels, while exposure to 5% O_2 alone induces profound hypocapnia (56). The mice were exposed to 20 H-IH episodes per hour for 7 h/day for 2, 7, or 21 days. Control animals were housed in similar cages with a constant flow of air. All animals were maintained on a 12:12-h light-dark cycle. SA patients experience 5–100 apneic episodes per hour of sleep.

Hemodynamic and activity recordings. Telemeter catheters were implanted using a modification of the procedure of Butz and Davisson as described previously (13). FVBN mice were given buprenorphine hydrochloride (Buprenex, 0.5 mg/kg sc) 20 min before surgery and anesthetized using isoflurane (5% supplemented with O_2). The catheter tip was inserted and secured in the carotid artery, and the transmitter body (model PA-C20, Data Sciences International) was secured subcutaneously above the right flank. Warmed sterile 0.9% NaCl solution (0.5 ml sc) was given after surgery, and the mice were allowed to recover for 5–7 days before recording was started. Mean arterial pressure (MAP), heart rate (HR), and activity were recorded daily for up to 36 days and analyzed using a telemetry analyzer (JCL Consultants). Each animal was used as a single *n* value.

As reported previously by Nieves-Cintron et al. (45), NFATc3 KO mice did not survive carotid artery catheter implantation surgery. We also observed that these mice do not survive femoral artery catheterization. Because breeding of this colony is very slow, systolic, diastolic, and mean blood pressures were monitored using the Coda 2 noninvasive blood pressure system (Kent Scientific, Torrington, CT) in the WT and NFATc3 KO mice immediately after the daily cycling. Animals were acclimated for 5 days before blood pressure was recorded. The Coda system utilizes volume-pressure-recording sensor technology for measurement of the mouse tail blood pressure parameters: systolic blood pressure, diastolic blood pressure, HR, mean blood pressure, tail blood flow, and tail blood volume. Volume-pressure recording has been clinically validated to provide close to 100% correlation with telemetry and direct blood pressure measurements for systolic and diastolic blood pressure (41, 50).

Assessment of polycythemia and left and right ventricular hypertrophy. Blood samples were obtained by direct cardiac puncture at the time of tissue collection for measurement of hematocrit. After isolation of the heart, the atria and major vessels were removed from the ventricles. The right ventricle (RV) was dissected from the left ventricle (LV) and septum. The degree of RV hypertrophy was expressed as the ratio of RV weight to total ventricular weight (RV + LV) plus septum weight, and LV hypertrophy was expressed as the ratio of LV weight to body weight.

Luciferase activity. After NFAT-luc mice were euthanized with an overdose of pentobarbital sodium (200 mg/kg ip), the aorta, brain and small intestines were removed and placed in ice-cold HEPES-physiological saline solution. The aorta and cerebral and mesenteric arteries were isolated and lysed using tissue lysis buffer (Promega) according to the manufacturer's protocol. The lysate was centrifuged for 10 min at 10,000 rpm. Luciferase activity and protein content were determined in the supernatant. Luciferase activity was measured using a luciferase assay system kit (Promega), and light was detected with a luminometer (model TD20/20, Turner). Protein content was determined by the Bradford method (Bio-Rad) and used to normalize luciferase activity.

Quantitative real-time PCR. LV and isolated arteries were stored in RNA*later* (Ambion). Total RNA was isolated using the RNeasy Mini Kit (Qiagen) following the manufacturer's protocol, with the addition of DNase Treatment (DNase Turbo, Ambion) and RNeasy MinElute Cleanup Kit (Qiagen) for removal of possible genomic DNA contamination. The High Capacity Reverse Transcription Kit (Applied Biosystems) was used for reverse transcription of total RNA to cDNA. For real-time PCR detection, SYBR Green Master Mix (Applied Biosystems) was used with the following sets of primer pairs: 5′-AGGTGCTGTCCCAGATGATTCTGT-3′ and 5′-TTGTGAGGCCTTGGTCCTTCAAGA-3′ for brain natriuretic peptide (BNP) in LV, 5′-GACATCATCTGGGTCAACACTC-3′ and 5′-CATCTAACTGCCTGGTCTGTG-3′ for prepro-ET-1, and 5′-TCAACTTTCGATGGTAGTCGCCGT-3′ and 5′-TCCTTGGATGTGGTAGCCGTTTCT-3′ for 18S RNA (endogenous control). PCR was performed using the model 7500 Fast Real-Time PCR System (Applied Biosystems). The normalized gene expression method ($2^{-\Delta\Delta C_T}$) was used for relative quantification of gene expression (40).

Immunofluorescence confocal microscopy. The number of NFATc3-positive nuclei was determined in aortic sections as previously described (11, 20, 27, 47, 48).

Statistical analysis. Values are means ± SE. Statistical significance was tested at 95% ($P < 0.05$) confidence level using unpaired *t*-test, one-way ANOVA followed by Tukey's multiple comparisons test, or two-way ANOVA followed by the Holm-Sidak test.

RESULTS

H-IH induces hypertension in mice. Animals exposed to H-IH showed a significant increase in MAP (Fig. 1*A*) on *day 1*, and this elevation was sustained up to the end of the experiment (*day 17*). This increase was observed during H-IH exposure and also during hours of air exposure. Interestingly, the normal fall in blood pressure during the day (sleep period) was inverted in H-IH-exposed mice. Activity also followed an inverted pattern (Fig. 1*B*), although no significant changes were observed in the level of activity.

Because of the change in diurnal blood pressure patterns, blood pressure during the hours of air exposure was no longer different between groups at the same time of day after 5 days of H-IH. However, it was different between groups when the comparison was made during the active hours (light for H-IH and dark for air) and during the inactive hours (dark for H-IH and light for air).

HR was not different at any time point (data not shown). Therefore, 7 h of H-IH daily during sleep appears to induce systemic hypertension in mice and disrupt normal activity patterns.

H-IH induces neither cardiac hypertrophy nor polycythemia in mice. Additional parameters were determined in mice exposed to air and H-IH for up to 21 days. H-IH-exposed mice did not develop polycythemia over the course of 21 days (Table 1). Normalized LV weight did not increase over the

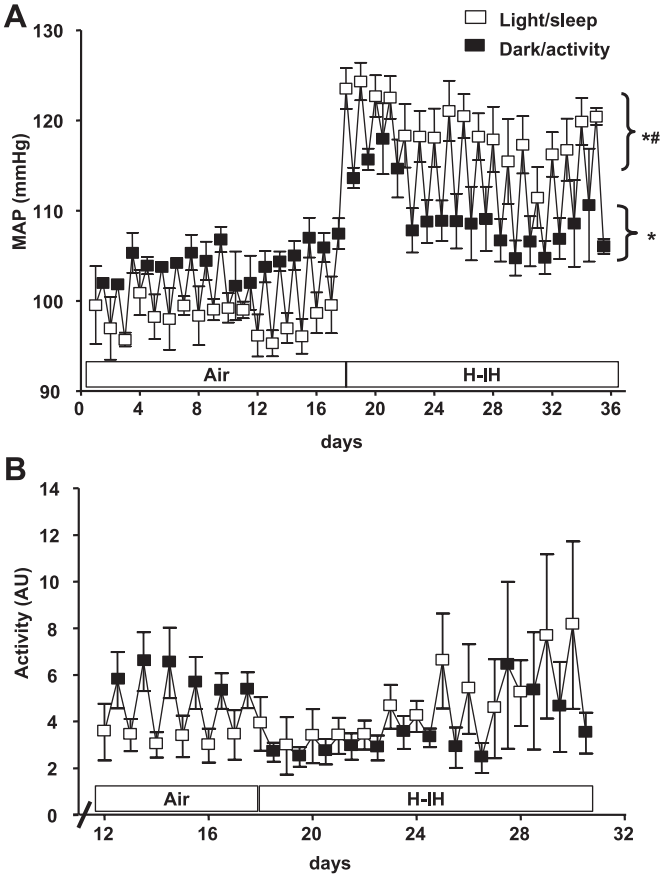

Fig. 1. Intermittent hypercarbic hypoxia (H-IH) induces hypertension in mice. *A*: mean arterial pressure (MAP) measured by telemetry in FVBN mice exposed to air cycling for 17 days followed by 18 days of H-IH cycling. MAP values were recorded from 7 AM to 7 PM (light/sleep) and from 7 PM to 7 AM (dark/activity) and averaged. Values are means ± SE ($n = 6$). *$P < 0.01$ vs. mean *days 1–17* light/sleep (97.1 ± 0.4 mmHg); #$P < 0.01$ vs. mean *days 1–17* dark/activity (104.3 ± 0.3 mmHg) by repeated-measures ANOVA and Holm-Sidak test. *B*: activity measured by telemetry in FVBN mice exposed to air cycling for 17 days followed by H-IH cycling for 18 days. Values recorded from *day 1* to *day 11* show the same pattern and, therefore, are not included. Values are means ± SE ($n = 6$). Note change in activity pattern, which is consistent with inverted diurnal/nocturnal pattern in MAP after H-IH. AU, arbitrary units.

period of H-IH exposure, suggesting no LV hypertrophy (Table 1). To further confirm that elevations in MAP were not associated with LV hypertrophy, BNP transcript levels were measured in LV from air- and H-IH-exposed mice at every time point. As shown in Table 1, no significant changes were observed in LV BNP mRNA compared with LV from air-exposed animals. In addition, no changes in the ratio of RV to RV + LV + septum weight (Table 1) were observed over the course of the experiment, suggesting no RV hypertrophy and pulmonary hypertension development.

H-IH induces prepro-ET-1 upregulation. Consistent with our previous observations in rats (35), H-IH induced a significant increase in mRNA levels of prepro-ET-1, the precursor of ET-1, in the lung (Fig. 2). Interestingly, prepro-ET-1 increased significantly after 2 and 7 days of H-IH and returned to air values at *day 21*.

H-IH induces calcineurin-mediated NFAT activation in aorta and mesenteric artery. Since NFAT has been shown to be activated in cerebral artery of ANG II-hypertensive mice (3, 45), we determined the degree of NFAT activation in aorta and cerebral and mesenteric arteries isolated from H-IH-exposed NFAT-luc mice. Luciferase activity, which reflects NFAT transcriptional activity, was significantly elevated in aorta and mesenteric artery after 2 and 7 days of H-IH (Fig. 3, *A* and *B*). In aorta, luciferase activity returned to air control values after 21 days of H-IH. Interestingly, there was a strong positive correlation between the elevation in aortic NFAT activity and prepro-ET-1 transcript levels in the lung (Fig. 3*C*). In mesenteric artery at *day 21*, luciferase activity tended to remain elevated compared with the air-exposed group, although the difference was not statistically significant ($P = 0.14$). In addition, a trend was observed for a correlation in the timing and degree of mesenteric artery NFAT activation and ET-1 upregulation, but it was not significant (Fig. 3*D*). These results are consistent with previous reports showing that ET-1 induces an increase in NFATc3 nuclear accumulation in vascular smooth muscle (25, 48). Taken together, these results suggest that ET-1 may be an upstream mediator of H-IH-induced NFAT activation in aorta and mesenteric artery.

Table 1. *H-IH induces neither cardiac hypertrophy nor polycythemia in mice*

	Air	H-IH 2 Days	H-IH 7 Days	H-IH 21 Days
%LV/BW	0.31±0.02	0.31±0.01	0.30±0.01	0.32±0.02
%RV/T	23.25±1.18	24.37±1.11	25.17±0.36	24.01±0.68
Fold change in LV BNP mRNA	0.84±0.19	0.87±0.17	1.19±0.39	0.89±0.09
Hematocrit	45±1	47±1	46±1	46±1

Values are means ± SE. FVBN mice were exposed to air and hypercarbia/hypoxia (H-IH) for ≤21 days. Heart was harvested, and blood was withdrawn by cardiac puncture. LV/BW, left ventricular (LV) weight-to-body weight ratio; RV/T, right ventricular (RV) weight-to-LV + RV + septum (T) weight ratio. Fold change in LV brain natriuretic peptide (BNP) mRNA was normalized by 18S RNA and measured by real-time PCR. Similar results were obtained in the other strain of mice.

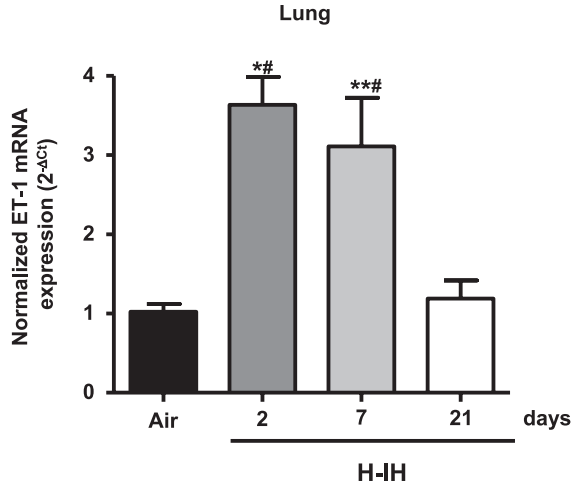

Fig. 2. H-IH induces upregulation of preproendothelin-1 (prepro-ET-1) transcripts in the lung. Prepro-ET-1 transcripts were measured by real-time PCR in total lung homogenates from 9×NFAT-luc mice exposed to air or H-IH for 2, 7, and 21 days. 18S RNA was used as an endogenous control. Values (means ± SE) are shown as fold increase vs. air ($n = 5$). *$P < 0.001$ vs. air; **$P < 0.01$ vs. air; #$P < 0.05$ vs. 21 days (ANOVA and Tukey's test).

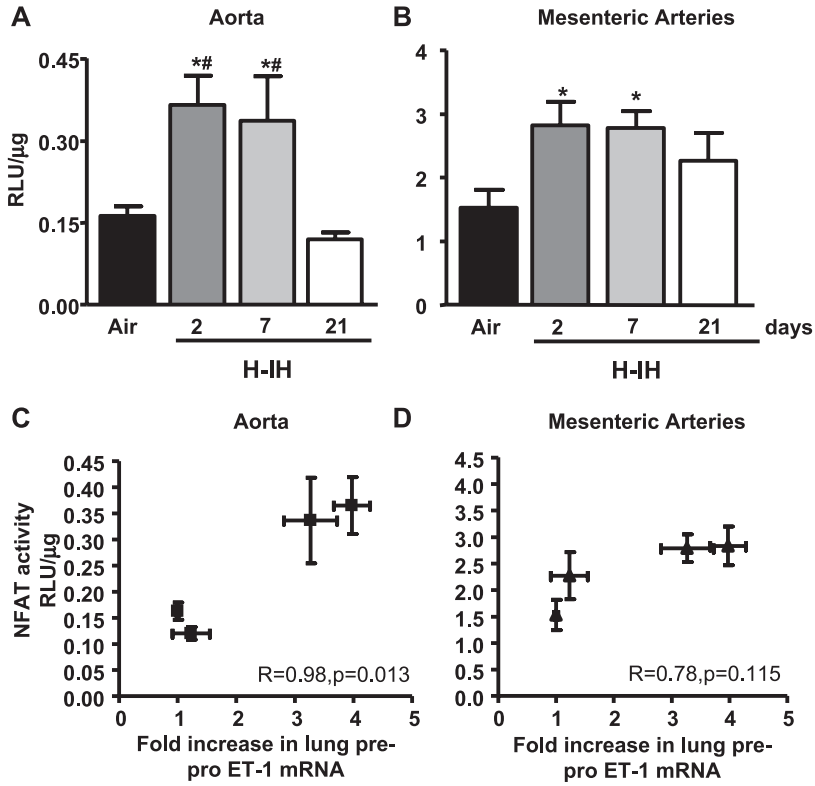

Fig. 3. Nuclear factor of activated T cells (NFAT) is activated by H-IH in the vasculature and correlates with prepro-ET-1 upregulation. *A* and *B*: luciferase activity in aorta and mesenteric arteries isolated from 9×NFAT-luc mice exposed to air or H-IH for 2, 7, and 21 days. Luciferase activity was normalized by micrograms of total protein. RLU, relative luciferase units. Values are means ± SE ($n = 6$). *$P < 0.01$ vs. air; #$P < 0.01$ vs. H-IH at 21 days (by ANOVA and Tukey's test). *C* and *D*: significant positive correlation between aortic NFAT activity and prepro-ET-1 mRNA lung levels and between mesenteric artery NFAT activity and prepro-ET-1.

NFAT was not significantly activated by H-IH in cerebral arteries (2.60 ± 0.43 in air and 2.87 ± 0.44, 3.22 ± 0.83, and 2.51 ± 1.10 at *days 2, 7*, and *21*, respectively, of H-IH). Interestingly, basal NFAT activity is higher in cerebral artery than in mesenteric artery and aorta: 2.60 ± 0.43 in cerebral artery ($P < 0.05$ vs. mesenteric artery and aorta), 1.53 ± 0.29 in mesenteric artery ($P < 0.05$ vs. cerebral artery and aorta), and 0.16 ± 0.02 in aorta ($n = 6$).

To address whether activation of calcineurin is upstream of NFAT activation, NFAT-luc mice were treated with CsA or vehicle for 3 days before and during 2 days of H-IH exposure. As expected, CsA completely prevented NFAT activation by H-IH in aorta and mesenteric artery (Fig. 4). These findings suggest that H-IH-induced NFAT activation requires calcineurin.

NFATc3 is the main isoform activated by H-IH. Since it has been previously demonstrated that NFATc3 significantly contributes to VSMC function (3, 26, 27), we sought to determine the contribution of this NFAT isoform to the H-IH-induced increase in NFAT activity. Therefore, we determined the effect of 2 days of H-IH on reporter expression in aorta and mesenteric artery isolated from NFAT-luc/NFATc3 WT and NFAT-luc/NFATc3 KO mice. Similar to results in Fig. 3, reporter activity increased in aorta and mesenteric artery after 2 days of H-IH in WT mice, but the reporter response to H-IH was significantly impaired in tissues isolated from NFAT-luc/NFATc3 KO mice (Fig. 5). NFATc3 nuclear accumulation consistently increased after H-IH exposure in aortic smooth muscle cells. Aortas from animals used in the experiments reported in Fig. 4 were used in Fig. 6 to show that 2 days of H-IH exposure increases aortic smooth muscle cell NFATc3 nuclear accumulation and that the increase is prevented by CsA.

These results support the conclusion that NFATc3 significantly contributes to H-IH-induced increases in NFAT transcriptional activity and further support our focus on this isoform.

Calcineurin-dependent NFATc3 activation mediates H-IH-induced hypertension. To determine the role of calcineurin/NFAT in H-IH-induced hypertension, animals were treated with CsA for 3 days before 7 days of H-IH exposure and MAP was recorded. Interestingly, CsA significantly attenuated the H-IH-induced increase in MAP (Fig. 7*A*) without affecting basal MAP during air exposure.

Since calcineurin does not exclusively regulate NFAT activity (54), we also measured changes in MAP in WT and NFATc3 KO mice exposed to H-IH for 7 days. Blood pressure was measured for 5 days of air exposure and at the end of the daily H-IH cycling for 7 days (both at the same time of day). Consistent with the results shown in Fig. 1, H-IH induced a significant and immediate increase in MAP in WT mice but failed to elevate MAP in NFATc3 KO mice. These results demonstrate that NFATc3 is required for the development of H-IH-induced systemic hypertension.

DISCUSSION

The present study demonstrates that H-IH induces systemic hypertension in mice and activates the Ca^{+2}/calcineurin-mediated transcription factor NFATc3 in aorta and mesenteric artery but does not cause concomitant cardiac hypertrophy or polycythemia. More importantly, inhibition of calcineurin or the genetic ablation of NFATc3 (NFATc3 KO) prevents H-IH-induced hypertension. In addition, prepro-ET-1 transcripts are elevated in the lung of mice exposed to H-IH, suggesting that it may be an upstream activator of NFATc3.

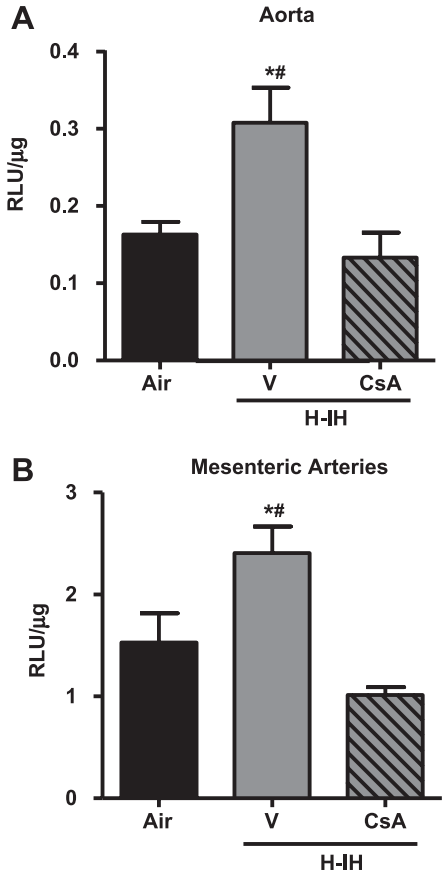

Fig. 4. Calcineurin inhibition prevents H-IH-induced NFAT activation. *A* and *B*: luciferase activity in aorta and mesenteric arteries isolated from air and H-IH-exposed 9×NFAT-luc mice treated with cyclosporin A (CsA, 25 $mg \cdot kg^{-1} \cdot day^{-1}$) or vehicle (V) for 3 days and exposed to H-IH for the last 2 days of CsA treatment. Luciferase activity was normalized by micrograms of total protein. Values are means ± SE ($n = 5$). *$P < 0.01$ vs. air; #$P < 0.01$ vs. CsA (ANOVA and Tukey's test).

Sleep-disordered breathing with recurrent apnea is a major cause of morbidity and mortality. Affected individuals have increased risk of systemic hypertension. To our knowledge, this is the first study reporting the development of systemic hypertension in a mouse model of SA, where mice are exposed to intermittent episodes of low O_2 (from 20.9 to 5%) and elevated CO_2 (from 0 to 5%). However, previous reports show that intermittent hypoxia (nadir O_2 of 5%) without CO_2 supplementation also induces systemic hypertension in mice (51, 58).

It is well established that nocturnal hypoxemia in SA patients causes an inversion of the circadian arterial pressure rhythm and triggers nocturnal hypertension (nondipping) (36, 62, 63). Consistently, our results show that mice exposed to H-IH show an inverted pattern in blood pressure and activity. In patients, it has been shown that nondipping hypertension is caused by cardiac preload, sleep disturbance, and hypoxia-induced increases in norepinephrine and ET-1 (62). Accordingly, we found that prepro-ET-1 transcripts are upregulated in the lung. Therefore, it is possible that the same mechanisms underlie the changes observed in our animal model.

Polycythemia is not consistently observed in SA animal models, with or without CO_2 supplementation, and SA patients (2, 9, 12, 16, 23, 30, 35, 42, 43, 56). In the present study, H-IH did not induce significant polycythemia in mice. Interestingly, our group recently demonstrated that polycythemia only develops in rats exposed to intermittent hypoxia without CO_2 supplementation (56), similar to other rat models (23, 35), where hematocrit does not increase in eucapnic intermittent hypoxia. Recently, Choi et al. (9) reported that SA patients show a very slight increase in hematocrit but do not develop clinical polycythemia, while others (2, 30) also suggested that SA patients do not develop polycythemia. These findings suggest differences in the physiological responses to intermittent hypoxia alone or with CO_2 supplementation.

Similar to alterations in hematocrit, the development of cardiac hypertrophy in intermittent hypoxia with or without CO_2 is also not consistent. Although several studies report that intermittent hypoxia induces modest-to-severe LV and RV hypertrophy in rodents (2, 8, 22, 23, 58), our study is in agreement with other reports showing that intermittent hypoxia with or without CO_2 supplementation does not induce cardiac hypertrophy (5, 17, 56). It is possible that LV hypertrophy did not develop in our mouse model because of CO_2 supplementation or that the elevation in systemic blood pressure was

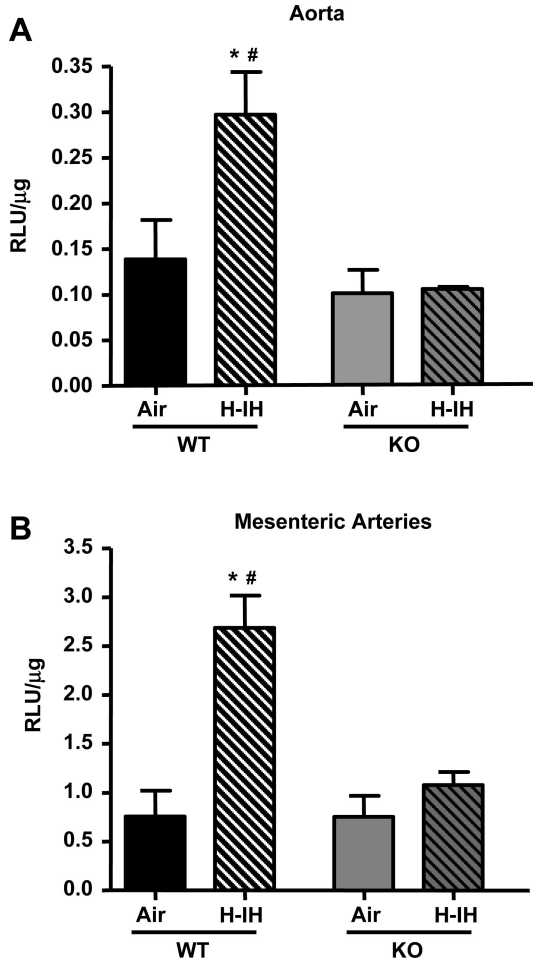

Fig. 5. NFATc3 is the main isoform activated by H-IH. Aorta (*A*) and mesenteric arteries (*B*) were isolated from 9×NFAT-luc/NFATc3 wild-type (WT) and knockout (KO) mice exposed to 2 days of H-IH. Luciferase activity was normalized by micrograms of total protein. Values are means ± SE ($n = 4–6$). *$P < 0.001$ vs. air; #$P < 0.001$ vs. KO (2-way ANOVA and Holm-Sidak test).

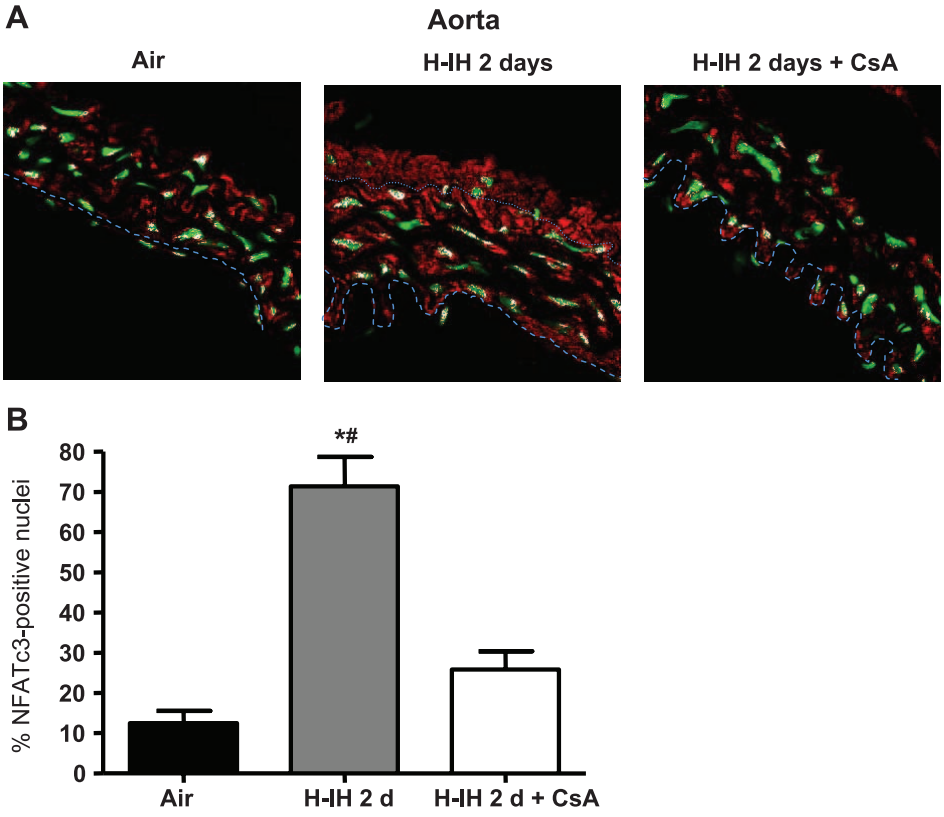

Fig. 6. H-IH induces NFATc3 nuclear accumulation in aortic smooth muscle cells. *A*: representative images showing cytosolic localization of NFATc3 under air and CsA conditions and nuclear localization following exposure to H-IH for 2 days. 9×NFAT-luc mice were treated with vehicle or CsA (25 $mg \cdot kg^{-1} \cdot day^{-1}$) and exposed to air or 2 days of H-IH. Sections of aorta were costained with the DNA-binding dye SYTOX (green) and anti-NFATc3 (red). Aortic smooth muscle nuclear colocalization of NFATc3 is shown in white. Dashed blue line shows limit of intimal and/or adventitial layer. Magnification ×40. *B*: summary of effects of CsA on H-IH-induced NFATc3 nuclear accumulation. Values are means ± SE (n = 8 images from ≥3 animals/group). *$P < 0.001$ vs. air; #$P < 0.001$ vs. H-IH.

below the threshold for activation of the cardiac hypertrophy gene program. Alternatively, proteolysis genes may have been turned on during H-IH to prevent hypertrophy, as suggested by Fan et al. (17).

The lack of RV hypertrophy and polycythemia in our mouse model suggests the absence of pulmonary hypertension, consistent with our recent results in rats exposed to intermittent hypoxia with 5% CO_2, which do not show RV hypertrophy, pulmonary arterial remodeling, or pulmonary hypertension (56). These results further support our focus on the systemic side of the circulation.

The present study shows for the first time that H-IH induces NFAT activation in small and large systemic vessels. This result is consistent with our previous report showing that NFATc3 nuclear accumulation increases in VSMC of ex vivo mouse arteries exposed to increased intraluminal pressure (27). Furthermore, Amberg et al. (3) and Nieves-Cintron et al. (45) showed that arterial NFATc3 is activated in ANG II-induced hypertension, independent of elevated blood pressure. Thus the elevated blood pressure during H-IH may not be the cause of the chronically increased NFATc3 activity. Our results suggest that ET-1 is one potential upstream activator of NFAT, since we found that transcripts of prepro-ET-1, the precursor of ET-1 and a potent activator of NFAT (25, 48), are elevated in the lung of mice exposed to H-IH. This tissue is a very important source of circulating ET-1 (14). In addition, it is well established that hypoxia upregulates ET-1 transcription through hypoxia-inducible factor-1α (15, 32, 33, 61) and that this pathway is activated by intermittent hypoxia (37). However, these studies do not exclude other factors, such as ANG II and/or catecholamines (10, 44, 52, 55), in the observed NFAT activation, particularly because heterozygous hypoxia-inducible factor-1α deficiency prevents intermittent hypoxia-induced hypertension and increased plasma norepinephrine (51).

NFAT activation in mesenteric arteries was sustained throughout the 21 days of H-IH, suggesting the importance of NFAT in resistance arteries. It is surprising that NFAT was not significantly activated in cerebral arteries from H-IH-exposed animals, since ET-1, ANG II, and catecholamines, potent stimuli for NFAT activation (1, 25, 27, 57), have been shown to be increased in H-IH (10, 44, 52, 55). However, blood flow control in the cerebral circulation is complex. Increased systemic blood pressure constricts large cerebral arteries with autoregulatory dilation of small vessels to maintain blood flow. In addition, hypoxia is a potent vasodilator in the cerebral circulation (for review see Ref. 19). Thus response to H-IH in the cerebral circulation may be very different from that in other systemic beds such as the mesentery. Alternatively, it is possible that NFAT is already maximally activated in cerebral arteries, since under basal conditions, luciferase levels are higher in cerebral artery than in mesenteric artery and aorta. This could be due to the elevated myogenic tone in this vascular bed under physiological conditions (18, 27), although it seems unlikely on the basis of reports of Amberg et al. (3) and Nieves-Cintron et al. (45), who showed that chronic administration of ANG II activates NFAT in the cerebral vasculature. Another explanation could be that the elevation of NFAT activators (ET-1, ANG II, and catecholamines) in the cerebral circulation is not sufficient to significantly activate NFAT.

NFATc3 is the isoform that seems to predominantly regulate the vasculature (3, 11, 20, 24–27, 45). Consistently, the present study showed that H-IH increases NFATc3 nuclear accumulation in aortic smooth muscle cells and that H-IH-induced

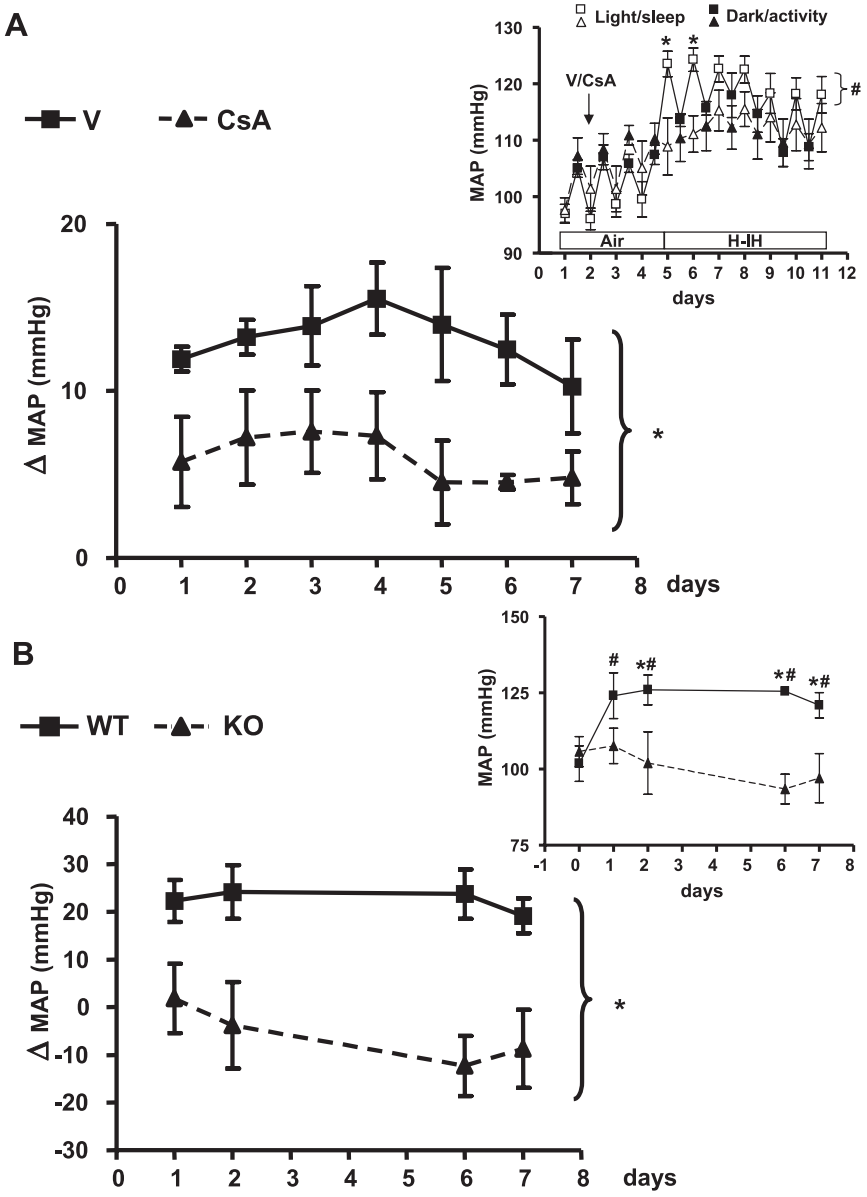

Fig. 7. NFAT inhibition prevents H-IH-induced hypertension. *A*: MAP (24 h) was measured by telemetry in FVBN mice injected with vehicle (V) or CsA (25 $mg \cdot kg^{-1} \cdot day^{-1}$ sc) for 3 days before H-IH and 7 days during H-IH. Data represent change from the mean of 3 days CsA only or V. Values are means ± SE ($n = 6$ for V and 4 for CsA). *$P < 0.05$ at every time point (2-way repeated-measures ANOVA and Holm-Sidak test). CsA alone did not affect MAP: 102.0 ± 1.5 and 109 ± 3.5 mmHg before and after CsA, respectively. *Inset*: MAP values separated into light/sleep (7 AM–7 PM) and dark/activity (7PM–7AM) periods and averaged. *$P <$ 0.05 vs. CsA; #$P < 0.01$ vs. light/sleep at *day 4* within V group; *day 7.5* (dark/activity) was different ($P < 0.05$) from *day 4.5* (dark/activity); no significant time effect was observed within the CsA group (2-way repeated-measures ANOVA and Holm-Sidak test). *B*: MAP was noninvasively measured by determination of tail blood volume with a volume-pressure-recording sensor and an occlusion tail cuff. MAP was measured in WT and NFATc3 KO mice exposed to air cycling for 3 days followed by 7 days of H-IH cycling. MAP was recorded at *days 1, 2, 6*, and *7* at the end of H-IH exposure. Data represent change from the mean of 3 days of air cycling. Values are means ± SE ($n = 5$). No significant difference was observed in baseline values between genotypes: 100.2 ± 7.3 and 109.6 ± 3.9 mmHg for WT and KO mice, respectively. *$P < 0.001$ (2-way repeated-measures ANOVA and Holm-Sidak test). *Inset*: raw MAP values, including those for the day before H-IH exposure (*day 0* = air exposure), for both genotypes. *$P < 0.05$ vs. KO; #$P < 0.05$ vs. *day 0* (2-way repeated-measures ANOVA and Holm-Sidak test).

NFAT activation is impaired in aorta and mesenteric artery from NFATc3 KO mice, supporting the importance of this isoform in the circulatory system and our focus on NFATc3. In addition, we recently demonstrated that NFATc3 is activated by hypobaric chronic hypoxia (an animal model of pulmonary hypertension) in mouse pulmonary arteries (11) and is required for the associated arterial remodeling. However, an elegant study by Bonnet et al. (6) shows that NFATc3 and NFATc2 are activated in pulmonary arterial smooth muscle cells from patients with secondary pulmonary hypertension and that NFATc2 is required for decreased apoptosis and increased pulmonary arterial remodeling in patients and monocrotaline-induced pulmonary hypertensive rats. Therefore, other NFAT isoforms might be important in rat and human pulmonary circulation, but our data strongly suggest that, in the peripheral vascular system of mice, NFATc3 is the most important isoform.

The most significant finding of our study is that H-IH-induced systemic hypertension is prevented by calcineurin inhibition with CsA and the genetic ablation of NFATc3, clearly demonstrating that NFATc3 is required for the development of hypertension in this model. These results are in agreement with the study of Nieves-Cintron et al. (45), which showed that NFATc3 is required for ANG II-induced hypertension, suggesting that this may be common in multiple forms of hypertension. Because previous reports demonstrate that activation of NFATc3 decreases the expression of voltage-dependent ($K_V2.1$) and large-conductance β-subunit K^+ channels in cerebral arteries contributing to hypertension, it is reasonable to speculate that NFATc3 may modulate the expression of these same genes to mediate enhanced vasoconstriction and hypertension during H-IH. This is further supported by our previous finding of increased ET-1-dependent vasoconstriction in mesenteric artery of H-IH-exposed rats (2, 56). Also, intermittent hypoxia has been shown by others to increase norepinephrine-dependent vasoconstriction (12, 39).

In conclusion, NFATc3 is activated in the systemic circulation by H-IH and is required for the development of H-IH-

induced hypertension. The present study highlights the potential clinical relevance of inhibition of the NFAT pathway to prevent the development of hypertension and its associated vascular pathology.

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