Augmented Endothelin Vasoconstriction in Intermittent Hypoxia-Induced Hypertension

Kyan J. Allahdadi, Benjimen R. Walker, Nancy L. Kanagy

Abstract—We reported previously that simulating sleep apnea in rats by exposing them 7 hours per day to intermittent hypoxia/hypercapnia (IH) elevates plasma endothelin-1 and causes hypertension, which is reversed by an endothelin-1 antagonist. We hypothesized that in this model of sleep apnea–induced hypertension, vascular sensitivity to endothelin-1 is increased in combination with the elevated plasma endothelin-1 to cause the endothelin-1–dependent hypertension. In small mesenteric arteries with endothelial function disabled by passing air through the lumen, diameter and vessel wall [Ca^{2+}] were recorded simultaneously. IH arteries demonstrated increased constrictor sensitivity to endothelin-1 (percentage max constriction 100±0% IH versus 80±10% Sham; P<0.05). This was accompanied by increased calcium sensitivity of IH arteries. In contrast, constrictor sensitivity and increases in vessel wall [Ca^{2+}] to KCl and phenylephrine were not different between IH and Sham arteries. We have shown previously that endothelin-1 constriction in mesenteric arteries is mediated by endothelin A receptors. In the current study, the selective increase in endothelin-1 constriction in IH resistance arteries was accompanied by increased expression of endothelin A receptor expression (densitometry units 271±23 IH versus 158±25 Sham; P<0.05). Thus, IH hypertension appears to cause alterations in signaling components unique to endothelin-1 at the receptor level and in postreceptor signaling that increases calcium sensitivity during endothelin A activation. Future studies will determine the specific changes in vascular smooth muscle signaling in IH hypertension causing this augmented contractile phenotype. (Hypertension. 2005;45:1-5.)

Key Words: rats ■ arteries ■ sleep apnea syndromes

Sleep apnea has been associated with many cardiovascular disease states, including the development of hypertension.1 Although the mechanism(s) by which sleep apnea causes hypertension are not clearly defined, there are many potential contributors to the development of cardiovascular disease. Studies have shown that sleep apnea is associated with sympathetic activation, hyperleptinemia, insulin resistance, elevated angiotensin II and aldosterone levels, oxidative and inflammatory stress, endothelial dysfunction, and impaired baroreflex function and effects on renal function.2 In addition, hypoxia per se increases plasma endothelin-1 (ET-1),3 and patients with sleep apnea have elevated circulating ET-1, which may also contribute to the chronic increase in arterial pressure.4 Indeed, previous studies have shown that rats exposed to intermittent hypoxia/hypercapnia (IH) to mimic the oxygen desaturation and CO2 accumulation that occurs during sleep apnea have increased circulating plasma ET-1 and develop ET-1–dependent hypertension.5 Thus, hypoxia- or stress-induced increases in ET-1 may be an important contributor to hypertension development in sleep apnea patients.

Chronic elevations in circulating ET-1 have been demonstrated in other models of hypertension.6,7 However, in studies in which vascular sensitivity to ET-1 has been evaluated, increases in circulating ET-1 are accompanied by desensitization and internalization of ET receptors.8,9 We demonstrated previously that depressor responses to ET-1 antagonists in IH-exposed rats causes a profound depressor response that is not seen in Sham rats.5 Thus, it is possible that unlike other models of ET-1–dependent hypertension, IH exposure leads to increases in ET-1 and ET-1–vascular sensitivity. Furthermore, the sympathetic nervous system has been suggested to play a major role in IH-induced hypertension as well,10 so that increases in vascular sensitivity to adrenergic constriction might also be augmented. This study was therefore designed to examine vascular responses to the adrenergic agonist phenylephrine (PE), depolarization-induced vasoconstriction by KCl, and constriction to ET-1. We hypothesized that small mesenteric arteries from IH rats would have a selective increase in constrictor sensitivity to ET-1 caused by alterations in signaling unique from that mediated by PE and KCl.

Methods

Animals

Male Sprague Dawley rats (weighing 250 to 300 g) were used for all studies and exposed to IH as described previously.5 Briefly, animals
were housed in Plexiglas boxes with free access to food and water and exposed to either IH at a rate of 20 cycles per hour (nadir 5% \(\text{O}_2\); 5% \(\text{CO}_2\) to peak 21% \(\text{O}_2\); 0% \(\text{CO}_2\)), or air–air cycling (alternating streams of 21% \(\text{O}_2\):0% \(\text{CO}_2\)) for 7 hours per day as described previously.\(^3\) A nadir of 5% \(\text{O}_2\) was chosen to simulate the systemic hypoxia observed in patients with severe sleep apnea, where minimum \(\text{O}_2\) saturations of \(<70\%\) are seen in patients with moderate to severe sleep apnea.\(^{11}\) In the current protocol, rats were exposed to \(P_{\text{O}_2}\) \(<10\%\) for approximately 1 minute of every 3-minute cycle. This level of hypoxia is similar to that used by other investigators to simulate IH in sleep apnea.\(^{12,13}\) Systolic blood pressure (SBP) and heart rate were recorded on days 0 and 14 to confirm the effect of the IH protocol on blood pressure. SBP was recorded before the start of the daily IH or air–air exposure using a standard tail–cuff apparatus (ITC). Body weight was also recorded to determine whether the exposure protocol altered weight gain. Approximately 16 hours after the final IH exposure, animals were deeply anesthetized with sodium pentobarbital (50 mg/kg) and mesenteric arteries collected for constrictor studies. Blood was also collected to measure hematocrit and hearts to measure left ventricle plus septum mass (left ventricle/body weight \([LV/BWT]\)) as an index of systemic hypertension-induced hypertrophy. All animal protocols were reviewed and approved by the institutional animal care and use committee of the University of New Mexico School of Medicine and conform to National Institutes of Health guidelines for animal use.

### Isolated Vessel Preparation

The intestinal arcade was removed and placed in a Silastic-coated Petri dish containing chilled physiological saline solution (PSS; [in mmol/L] 129.8 NaCl, 5.4 KCl, 0.83 MgSO\(_4\), 19 NaHCO\(_3\), 1.8 CaCl\(_2\), and 5.5 glucose). Fourth-order artery segments were dissected from the mesenteric vascular arcade and placed in fresh PSS oxygenated with normoxic gas (21% \(\text{O}_2\):6% \(\text{CO}_2\):73% N\(_2\)). Cleaned arterioles were transferred to a vessel chamber (Living Systems), cannulated with glass micropipettes, and secured with silk ligatures. The vessels were slowly pressurized to 60 mm Hg with PSS using a servo-controlled peristaltic pump (Living Systems) and superfused with oxygenated 37°C PSS at a rate of 5 mL per minute.

### Endothelium Removal

The endothelium was disabled in all experiments by passing 1 mL of air through the lumen. The integrity of the endothelium was assessed before and after denuding by exposing PE (10 \(\mu\)mol/L)-contracted arterioles to acetylcholine (1 \(\mu\)mol/L). Acetylcholine-mediated vasodilation was eradicated in vessels successfully disabled.

### Fura 2-Acetoxymethyl Ester Loading

After denuding, pressurized mesenteric arteries were loaded with the cell-permeable ratiometric Ca\(^{2+}\)-sensitive fluorescent dye fura 2-acetoxymethyl ester (fura 2-AM; Molecular Probes). Fura 2-AM was dissolved in anhydrous dimethyl sulfoxide (DMSO; 1 \(\mu\)mol/L). Directly before loading, fura 2-AM was mixed with a 20% solution of pluronic acid in DMSO, then added to PSS yielding an end concentration of 2 \(\mu\)mol/L fura 2-AM and 0.05% pluronic acid. Pressurized arterioles were incubated 45 minutes in the dark at room temperature in oxygenated fura 2-AM solution. After incubation, arteries were washed with 37°C PSS for 15 minutes to remove excess dye and allow complete esterification of the compound. Fura 2–loaded vessels were alternately excited at 340 and 380 nm at a frequency of 10 Hz with an IonOptix Hyperswitch dual-excitation light source and the respective 510-nm emissions collected with a photomultiplier tube (F\(_{340}/\text{F}_{380}\)). Background-subtracted F\(_{340}/\text{F}_{380}\) emission ratios were calculated with Ion Wizard software (IonOptix) and recorded continuously throughout the experiment with simultaneous measurement of inner diameter from bright-field images as described previously.\(^{14}\)

### Constrictor Studies

After determining baseline internal diameter and F\(_{340}/\text{F}_{380}\) constrictions were produced by exposing arterioles to increasing concentra-

### Physiological Parameters for Test Groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Hematocrit (%)</th>
<th>LV/BWT ((\times 1000))</th>
<th>SBP (mm Hg)</th>
<th>Body Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 0</td>
<td>—</td>
<td>—</td>
<td>134.4 ± 5.5</td>
<td>283 ± 5</td>
</tr>
<tr>
<td>Day 14</td>
<td>44.9 ± 0.677</td>
<td>2.11 ± 0.03</td>
<td>134.7 ± 5.3</td>
<td>324 ± 5</td>
</tr>
<tr>
<td>IH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 0</td>
<td>—</td>
<td>—</td>
<td>133.9 ± 5.5</td>
<td>285 ± 3</td>
</tr>
<tr>
<td>Day 14</td>
<td>47.7 ± 0.4*</td>
<td>2.24 ± 0.04*</td>
<td>153.3 ± 6.5†</td>
<td>308 ± 4†</td>
</tr>
</tbody>
</table>

*Significant difference from Sham for \(P<0.05\); †significant difference from day 0 within group.

### Western Blot Analysis

To determine whether IH alters expression of vascular ET\(_A\) and ET\(_B\) receptors, protein levels were evaluated as described previously.\(^4\) Briefly, mesenteric artery cascades from Sham and IH rats were cleaned of connective and adipose tissue, frozen in liquid nitrogen, and homogenized on ice in Tris–HCl buffer (pH 7.4) containing protease inhibitors. Homogenates were centrifuged 1500g at 4°C for 10 minutes to remove insoluble material. After determining supernatant protein concentration by the Bradford method (Pierce protein assay), 25 \(\mu\)g protein was separated in 4% to 20% gradient polyacrylamide gels (BioRad) and transferred to polyvinylidifluoride membranes. After blocking, membranes were incubated for 2 hours at 25°C, then overnight at 4°C with a rabbit monoclonal antibody specific for rat ET\(_A\) (1:2500) or ET\(_B\) (1:5000; Biodesigns, Inc.) in TBS with 0.05% Tween-20. After washing, blots were incubated for 1 hour with peroxidase-labeled goat anti-rabbit IgG (1:5000; Bio-Rad), followed by chemiluminescence labeling (enhanced chemiluminescence assay; Amersham). ET\(_A\) and ET\(_B\) band densities (SigmaGel; SPSS) were normalized to the total protein loaded per lane as determined by Coomassie staining of the membranes.

### Statistical Analysis

Constriction and Ca\(^{2+}\) concentration response curves were analyzed using 2-way repeated-measures ANOVA with Student-Newman–Keuls post hoc analysis for differences between groups, concentrations, and interactions. Percent data were transformed to the square root of the arctan before analysis to assure normalized data (SigmaStat Software; SPSS). Covariate analysis of fura-2 ratios and percentage vasconstriction were further analyzed to determine the dependence of constriction on vessel wall [Ca\(^{2+}\)]. Calculated slope and y-intercept values were analyzed using SASS statistical analysis software. \(P<0.05\) was considered statistically significant for all analyses.

### Results

**Blood Pressure**

Blood pressure was significantly increased in IH rats on day 14 compared with day 0 and compared with Sham rats on day 14 (Table). LV/BWT and hematocrit were significantly elevated in IH rats compared with Sham rats on day 14.
Although both groups had a significant increase in body weight between day 0 and day 14, body weight in IH rats on day 14 was significantly less than that in Sham rats.

**Contractile Studies**

Concentration-dependent responses to ET-1, PE, and KCl in IH and Sham arteries are expressed as percentage vasoconstriction from baseline, with 100% being a completely closed lumen. IH arteries had augmented constrictor sensitivity to ET-1 compared with Sham arteries demonstrated as a leftward shift in the curve (Figure 1A). PE and KCl constrictions were not different between IH and Sham arteries (Figure 1B and 1C).

**Vessel Wall [Ca^{2+}]**

Changes in vessel wall [Ca^{2+}] were determined as the F_{340/380} measured simultaneously with inner diameter. In contrast to the difference in constriction for ET-1, F_{340/380} were not different between groups (Figure 2A), although they did approach significance as indicated by the P values in Figure 2. Similarly, PE and KCl F_{340/380} responses were not different between groups (Figure 2B and 2C). Interestingly, in the Sham arteries, all agents increased vessel wall [Ca^{2+}] simultaneous with and parallel to vasoconstriction. However, in the IH arteries, only PE and KCl caused concentration-dependent increases in [Ca^{2+}]. Thus, ET-1 constriction in IH arteries was not accompanied by changes in vessel wall [Ca^{2+}], and the ET-1 constriction in the IH arteries appears to be caused by mechanisms independent of increases in [Ca^{2+}].

**Calcium Sensitivity**

When vasoconstriction was plotted as a function of vessel wall [Ca^{2+}], constriction at any given intracellular [Ca^{2+}] was greater in IH arteries than in Sham arteries in the presence of ET-1 (Figure 3A). In contrast, constrictions to PE and KCl occurred in the presence of similar increases in [Ca^{2+}] in the IH and Sham arteries. Thus, ET-1 constriction appears to rely more on increased calcium sensitivity in IH arteries than in Sham arteries, whereas PE and KCl produce constrictions with similar increases in [Ca^{2+}] and calcium sensitivity in the 2 groups. This suggests IH exposure causes a specific alteration in the ET-1 signaling pathway.

**Receptor Expression**

To determine whether the increased constrictor response to ET-1 in IH mesenteric arteries was attributable to ET receptor upregulation, ET_{A}R and ET_{B}R receptor protein expression was evaluated. Western analysis using commercially distributed antibodies produced blots with a single band migrating at 63 kDa for ET_{A}R and a doublet at 48 kDa for ET_{B}R, as has been reported previously in vascular tissue.9 Densitometric analysis of blots normalized to the density of Coomassie blue staining indicated that expression of ET_{A}R was increased, but expression of ET_{B}R was not different between groups. These data suggest there is increased expression of ET_{A}R protein but no change in ET_{B}R protein in IH arteries (Figure 4). This increased receptor expression may explain in part the augmented ET-1 constriction.
Discussion

When ET-1 production is increased by hypoxia or other stimuli, there can be a reciprocal downregulation of endothelin receptors. Thus, IH-induced increases in circulating ET-1 could lead to decreased vascular sensitivity to the peptide. However, we reported previously that acute administration of an ET-1 antagonist causes a decrease in blood pressure in IH rats but not in Sham rats. Thus, we hypothesized that IH exposure might cause a simultaneous increase in circulating ET-1 and vascular sensitivity to the peptide. We tested this hypothesis by examining vascular reactivity to ET-1 and other vasoconstrictors in small mesenteric arteries.

Key findings in this study are: (1) arteries from hypertensive IH rats have augmented vasoconstrictor responses to ET-1 compared with arteries from Sham rats; (2) when vasoconstriction is plotted as a function of vessel wall [Ca$^{2+}$], IH arteries constrict more for any given [Ca$^{2+}$] in the presence of ET-1; (3) IH arteries do not have augmented calcium sensitivity compared with Sham arteries in the presence of PE and KCl; and (4) IH arteries express more ETA receptors than Sham arteries. These data indicate that IH exposure selectively augments ET-1 vasoconstriction independent of increased calcium signaling and suggest IH causes a selective alteration in ET-1 signal transduction in vascular smooth muscle cells independent of calcium influx pathways and unique from signaling pathways used by PE and KCl.

Our observation that ET$\alpha$R expression is increased in IH arteries compared with Sham although ET$\beta$R expression is unchanged indicates that IH exposure upregulates rather than downregulates ET$\beta$R expression. Glucocorticoids transcriptionally upregulate preproendothelin and ET$\alpha$R. Therefore, stress-induced increases in glucocorticoid production during IH exposure may contribute to the elevated ET-1 and ET$\alpha$R expression. However, ET-1 constriction in IH arteries is independent of increases in vessel wall [Ca$^{2+}$], whereas that in Sham arteries is accompanied by increased [Ca$^{2+}$]. Thus, the augmented response in the IH arteries does not appear to be caused by activation of more receptors. Rather, ET$\alpha$R activates a different pathway in the IH arteries that is linked exclusively to elevated calcium sensitivity. These data suggest that the mechanism of ET-1–mediated constriction is altered at a postreceptor site in the IH arteries.

ET-1 vasoconstrictor responses were compared with KCl and PE to clarify potential points of upregulation in the ET-1 signaling pathway. KCl, an electro-mechano–coupling agent, causes vasoconstriction by decreasing the driving force for K$^+$, ultimately depolarizing vascular smooth muscle cells. This depolarization activates voltage-gated calcium channels leading to Ca$^{2+}$ influx. Conversely, PE is a pharmacomechano–coupling agent that causes vasoconstriction by activating the G-protein–coupled $\alpha_1$-adrenergic receptor. Thus, a nonspecific increase in calcium sensitivity or in calcium channel function should augment all 3 responses, whereas an increase in receptor-activated second messenger responses common to ET$\alpha$ and $\alpha_1$adrenergic receptors would augment PE and ET-1 constrictions. The increased sensitivity to only ET-1 indicates that the alteration in IH arteries is unique to the ET-1 pathway.

ET$\alpha$ receptors act primarily through G$\alpha$ proteins and cause constriction by increasing intracellular [Ca$^{2+}$] and vasoconstrictor sensitivity to calcium. In the present study, ET-1 caused a concentration-dependent increase in [Ca$^{2+}$], that paralleled constriction in Sham arteries. However, in IH arteries, constriction occurred in the absence of an increase in [Ca$^{2+}$]. This suggests calcium handling is altered in the IH

---

**Figure 3.** Vasoconstriction plotted as a function of vessel wall calcium (F$340$/F$380$) for ET-1 (A), PE (B), and KCl (C). Increases in tone were significantly dependent on [Ca$^{2+}$] for all curves except ET-1 in IH arteries as evaluated by analysis of covariance. *Significant difference in slope and $y$ intercept from Sham arteries for $P<0.05$.

**Figure 4.** Expression of ET$\alpha$R (A) and ET$\beta$R (B). Blots were densititized and normalized for protein loading. Bar graphs illustrate mean normalized densitometry units. *Significant difference between IH and Sham for $P<0.05$. 

---

*Figure 3.* [Graph showing vasoconstriction plotted as a function of vessel wall calcium (F$340$/F$380$) for ET-1 (A), PE (B), and KCl (C). Increases in tone were significantly dependent on [Ca$^{2+}$] for all curves except ET-1 in IH arteries as evaluated by analysis of covariance. *Significant difference in slope and $y$ intercept from Sham arteries for $P<0.05$.]

*Figure 4.* [Expression of ET$\alpha$R (A) and ET$\beta$R (B). Blots were densititized and normalized for protein loading. Bar graphs illustrate mean normalized densitometry units. *Significant difference between IH and Sham for $P<0.05$.]
arteries. ET-1 increases \([\text{Ca}^{2+}]\), by efflux from the sarcoplasmic reticulum and by influx through membrane channels.\(^{16,17,20,21}\) Furthermore, ET-1 can cause constriction by increasing calcium sensitivity via Rho kinase and protein kinase C activation, with subsequent inhibition of myosin light chain phosphatase.\(^{18}\) This study indicates that ET-1 constriction of arteries from IH rats is independent of \([\text{Ca}^{2+}]\), changes. Thus, 14 days of exposure to IH alters ET\(_R\) signaling in small mesenteric arteries, increasing activation of calcium-sensitizing pathways and decreasing activation of \([\text{Ca}^{2+}]\) influx. Furthermore, although similar intracellular signaling pathways can be activated by \(\alpha_1\)-adrenergic and ET\(_A\) receptors,\(^{18,22}\) there is clearly an ET-1–exclusive pathway that is altered by IH exposure. Although only 1 vascular bed was examined, small mesenteric arteries are good models of resistance arteries with dense innervation and significant contributions to systemic vascular resistance.\(^{23,24}\) Thus, selective vascular reactivity to ET-1 in resistance arteries may explain a portion of sleep apnea–induced hypertension

### Perspectives

IH-exposed rats have increased circulating ET-1 similar to that reported in sleep apnea patients,\(^2\) suggesting hypertension in IH rats simulates at least some aspects of this medical condition. Patients with sleep apnea also have elevated sympathetic activation, increased daytime blood pressure, and increased risk of cardiovascular morbidity. Thus, the novel observation in this study that increases in vascular sensitivity to ET-1 are present in an animal model of sleep apnea–induced, ET-1–dependent hypertension\(^5\) highlights the potential benefit of blocking this pathway to prevent the cardiovascular morbidity common in sleep apnea patients.\(^{2,25,26}\)

### Acknowledgments

This study was funded by NHLBI 03852 (N.L.K.), HL63207 (B.R.W.), T32 HL007736 (K.J.A.), a grant-in-aid from the Desert Mountain Affiliate of the American Heart Association, and center funds from National Institute of Environmental Health Sciences grant P30 ES-012072. N.L.K. is an established investigator of the American Heart Association.

### References
