Reactive oxygen species contribute to sleep apnea-induced hypertension in rats

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Troncoso Brindeiro CM, Silva AQ, Allahdadi KJ, Youngblood V, Kanagy NL. Reactive oxygen species contribute to sleep apnea-induced hypertension in rats. Am J Physiol Heart Circ Physiol 293: H2971–H2976, 2007. First published August 31, 2007; doi:10.1152/ajpheart.00219.2007.—In clinical studies, sleep apnea is associated with hypertension, oxidative stress, and increased circulating endothelin-1 (ET-1). We previously developed a model of sleep apnea by exposing rats to eucapnic intermittent hypoxia (IH-C) during sleep, which increases both blood pressure and plasma levels of ET-1. Because similar protocols in mice increase tissue and plasma markers of oxidative stress, we hypothesized that IH-C generation of reactive oxygen species (ROS) contributes to the development of ET-1-dependent hypertension in IH-C rats. To test this, male Sprague-Dawley rats were instrumented with indwelling blood pressure telemetry and drank either plain water or water containing the superoxide dismutase mimetic, Tempol (4-hydroxy-2,2,6,6-tetramethyl-piperidine-1-oxyl, 1 mM). Mean arterial pressure (MAP) and heart rate (HR) were recorded for 3 control days and 14 treatment days with rats exposed 7 h/day to IH-C or air/air cycling (Sham). On day 14, MAP in IH-C rats treated with Tempol (107 ± 2.29 mmHg) was significantly lower than in untreated IH-C rats (118 ± 9 mmHg, P < 0.05). Tempol did not affect blood pressure in sham-operated rats (Tempol = 101 ± 3, water = 101 ± 2 mmHg). Immunoreactive ET-1 was greater in plasma from IH-C rats compared with plasma from sham-operated rats but was not different from Sham in Tempol-treated IH-C rats. Small mesenteric arteries from IH-C rats but not Tempol-treated IH-C rats had increased superoxide levels as measured by ferric cytochrome c reduction, lucigenin signaling, and dihydroethidium fluorescence. The data show that IH-C increases ET-1 production and vascular ROS levels and that scavenging superoxide prevents both. Thus oxidative stress appears to contribute to increases in ET-1 production and elevated arterial pressure in this rat model of sleep apnea-induced hypertension.

Tempol; endothelin

The repeated episodes of cessation of breathing that occur during sleep apnea have been linked to the increased generation of reactive oxygen species (ROS) with an associated increase in cardiovascular morbidity (1). Since it is estimated that up to one in five adults in Western countries suffer from sleep apnea (36), this condition appears to be a significant cause of cardiovascular disease. Several longitudinal studies of sleep apnea patients conclude that the greatest health risk of sleep apnea is the increased incidence of cardiovascular disease (24, 42), with the most consistent alteration being increased mean arterial pressure (MAP) (26, 30). Thus almost 50% of people suffering from sleep apnea also suffer from hypertension (13), and it is of great relevance to better understand the role of oxidative stress in sleep apnea-associated hypertension.

Repeated apneic episodes are reported to increase oxidative stress in sleep apnea patients (16, 21), in animal models of sleep apnea (31, 41), and in cultured cells exposed to cyclical hypoxia (43). However, debate continues on the consequences of this elevated oxidative stress. One potential consequence is an increased production of the potent vasoconstrictor, endothelin 1 (ET-1), which can be induced by ROS (18). A role for ET-1 in sleep apnea-associated cardiovascular disease is further suggested by increased circulating plasma ET-1 (32, 44), which is reversed when apneic episodes are prevented with continuous positive airway pressure (17). Previously, we have shown that in rats with simulated sleep apnea, circulating plasma ET-1 increases in parallel with increases in arterial pressure and that blocking ET-1 receptors lowers MAP in these rats (20). Therefore, the mechanistic link between sleep apnea, increased ET-1 synthesis, and hypertension may be oxidative stress.

Free radicals have previously been shown to play a role in endothelial dysfunction and ET-1 synthesis in hypertension. Kaecher et al. (18) and Cheng et al. (4, 5) have reported that ROS, specifically superoxide, increase prepro-ET-1 promoter activity. We therefore hypothesized that ET-1 production is stimulated by increased ROS generation during the intermittent periods of hypoxia associated with sleep apnea, leading to ET-dependent systemic hypertension.

We tested this hypothesis by measuring ROS in arteries from eucapnic intermittent hypoxia (IH-C) and sham-operated rats and by scavenging ROS with the antioxidant, Tempol (4-hydroxy-2,2,6,6-tetramethyl-piperidine-1-oxyl, a superoxide dismutase mimetic) during IH-C treatment in rats and by determining the effect on blood pressure and circulating ET-1.

MATERIALS AND METHODS

Animal model. Animal protocols for this study were approved by the Institutional Animal Care and Use Committee of the University of New Mexico and follow the National Institutes of Health guidelines for animal use in research. Male Sprague-Dawley rats (200–250 g body wt; Harlan, Indianapolis, IN) were instrumented with indwelling
blood pressure telemeters (DSI model PA-C40; Arden Hills, MN), allowing daily recording of MAP and heart rate (HR). Telemeter devices were sutured to the abdominal cavity wall, and the catheter tip was advanced into the abdominal aorta via the femoral artery. Control hemodynamic recordings were initiated 5 days after the surgery.

**Intermittent hypoxia/eucapnia protocol.** After recovery, rats (n = 26) were randomly divided into either IH-C (n = 15) or air-exposed (Sham; n = 13) groups. Sham animals were exposed to the same environmental factors as IH-C, except the gas in the chamber was always air (21% O2). Vehicle-treated IH-C (n = 9) and Sham rats (n = 7) drank tap water, whereas Tempol-treated rats drank water containing Tempol throughout the treatment period (1 mM, n = 6/group). This dose of Tempol has previously been shown to prevent increases in oxidative stress in ET-1-infused rats (34, 38). The atmosphere in the IH-C chamber was controlled by electronically regulated solenoid switches in a three-channel gas mixer, which gradually lowered oxygen in the chamber over 90 s from 21% to ~5% O2 and increased the CO2 content from 0% to ~5% (Technolutions). Chambers were rapidly flushed with room air for the following 90 s to restore O2 to ~21% and CO2 to 0%. Rats were exposed to 20 IH-C cycles/h for 7 h/day during their sleep period for 14 days.

**Measurement of superoxide with ferricytochrome c.** The amount of superoxide generated in mesenteric arterioles was measured by the reduction of ferricytochrome c using a kinetic spectrophotometer (DU 530; Beckman, Fullerton, CA). Reduction of ferricytochrome c by O2- increases absorbance at 550 nm and can be used as a quantitative measure of O2- production (33). Absorbance at 550 nm was recorded every minute for 10 min before and 35 min after addition of a freshly isolated single mesenteric vascular tree to PBS containing ferricytochrome c (5 x 10^-5 M, RT). A second branch of approximately equal weight (0.49 ± 0.02 mg average wt) was added to a second cuvette containing the SOD mimetic, tiron (10 μM). The difference in A550 in the absence and presence of tiron was used to estimate O2- generation/arteriole segment.

**Lucigenin assay.** Small mesenteric arteries were collected from rats anesthetized with pentobarbital sodium (150 mg/kg ip) on day 14 of treatment. Arteries were placed in cold physiological saline solution (PSS), containing in mM) 129.8 NaCl, 5.4 KCl, 0.83 MgSO4, 19 NaHCO3, 1.8 CaCl2, and 5.5 glucose and then transferred to a test tube containing lucigenin in HEPES-buffered saline (1 mmol, pH 7.4, room temperature). Background luminescence of the solution without arteries was subtracted from basal and NADPH-stimulated (0.1 mM) luminescence for each sample. Measurements were normalized for the dry weight of arteries.

**Dihydroethidium staining.** The superoxide-sensitive fluorescent dye, dihydroethidium (DHE), was used to evaluate in situ production of superoxide. DHE is freely permeable to cells and, in the presence of superoxide. DHE is freely permeable to cells and, in the presence of superoxide, DHE is freely permeable to cells and, in the presence of superoxide, DHE is freely permeable to cells and, in the presence of superoxide. DHE is freely permeable to cells and, in the presence of superoxide, DHE is freely permeable to cells and, in the presence of superoxide, DHE is freely permeable to cells and, in the presence of superoxide, DHE is freely permeable to cells and, in the presence of superoxide, DHE is freely permeable to cells and, in the presence of superoxide, DHE is freely permeable to cells and, in the presence of superoxide, DHE is freely permeable to cells and, in the presence of superoxide, DHE is freely permeable to cells and, in the presence of superoxide, DHE is freely permeable to cells and, in the presence of superoxide, DHE is freely permeable to cells and, in the presence of superoxide, DHE is freely permeable to cells and, in the presence of superoxide, DHE is freely permeable to cells and, in the presence of superoxide, DHE is freely permeable to cells and, in the presence of superoxide, DHE is freely permeable to cells and, in the presence of superoxide, DHE is freely permeable to cells and, in the presence of superoxide, DHE is freely permeable to cells and, in the presence of superoxide, DHE is freely permeable to cells and, in the presence of superoxide, DHE is freely permeable to cells and, in the presence of superoxide, DHE is freely permeable to cells and, in the presence of superoxide, DHE is freely permeable to cells and, in the presence of superoxide, DHE is freely permeable to cells and, in the presence of superoxide, DHE is freely permeable to cells and, in the presence of superoxide, DHE is freely permeable to cells and, in the presence of superoxide, DHE is freely permeable to cells and, in the presence of superoxide, DHE is freely permeable to cells and, in the presence of superoxide, DHE is freely permeable to cells and, in the presence of superoxide, DHE is freely permeable to cells and, in the presence of superoxide, DHE is freely permeable to cells and, in the presence of superoxide, DHE is freely permeable to cells. DHE staining was performed 24 days after the surgery.

**RESULTS**

**Hemodynamic measurements.** MAP and HR were not different during the baseline recording period between the four groups. However, after 14 days of treatment, there was a significant difference in MAP between the IH-C/vehicle group (118 ± 8.8 mmHg) and the other three groups: Sham/vehicle (101 ± 1.7 g), Sham/Tempol (101 ± 3.1 g), and IH-C/Tempol (107 ± 2.3 mmHg) (Fig. 1). In the Tempol-treated IH-C rats, MAP was not significantly different from that in the Sham groups. Heart rate did not differ between any of the four groups, did not change over the time of treatment, and was not different between groups at day 14: Sham/vehicle 392 ± 3.5, Sham/Tempol 372 ± 38.7, IH-C/vehicle 395 ± 3.1, and IH-C/Tempol 391 ± 14.8 beats/min.

**Superoxide generation.** Superoxide (O2-) generation measured by cytochrome c reduction was significantly greater in arteries from the IH-C group compared with the Sham group (Fig. 2). There was no difference between arteries from Sham and IH-C rats treated in vivo with Tempol. 

**ROS-activated luminescence.** Basal measurements of ROS, using lucigenin as a detector, were significantly greater in arteries from the IH-C arteries than in the Sham arteries when normalized for tissue dry weight (luminescence/tissue wt in mg). NADPH stimulated a large increase in luminescence.
ence in both groups, and the difference between groups was even greater than in the absence of NADPH (Fig. 3).

**DHE staining.** Arteries from IH-C rats had greater DHE-induced fluorescence than arteries from Sham rats (Fig. 4). Fluorescence in arteries from IH-C rats treated with Tempol was not different from that in Sham arteries. This indicates that there is greater ROS generation in the vascular wall following 14 days of IH-C exposure but that the increase is prevented by in vivo Tempol treatment.

**Plasma ET-1.** The calculated concentration of immunoreactive ET-1 in plasma samples from the IH-C rats was greater than that in the Sham rat samples (Fig. 5). However, the concentration in the samples from the IH-C rats drinking Tempol-containing water was not different from that in the Sham rats. Plasma ET-1 was also not different between Sham rats drinking water and those drinking water with Tempol. Thus IH-C increases circulating ET-1, and Tempol specifically reduces ET-1 in IH-C rats. This suggests that Tempol prevents IH-C-induced hypertension, in part, by decreasing ET-1 synthesis.

**DISCUSSION**

Sleep apnea is a growing health problem in the United States, and many studies have reported a significant association between cardiovascular disease and sleep apnea (7, 14, 15). Hypertension has the strongest correlation with 50% of sleep apnea patients also having hypertension (25). Because the increased risk of mortality in patients with obstructive sleep apnea (OSA) is largely from cardiovascular incidents, understanding the mechanisms underlying the cardiovascular pathology of this condition will allow more directed interventions to prevent permanent or lethal damage. Although corrective therapies to prevent the repeated apneas during sleep reduce the increased cardiovascular complications of OSA, compliance...
tion-induced hypocapnia (22). This is in contrast to studies by
Fletcher and coworkers (12, 22), who saw no chronic effect on
blood pressure by supplementing IH with CO2, a protocol
similar to ours that maintains eucapnia (22). However, in the
previous studies, the exposure protocol had very rapid IH
cycling (2 cycles/min), and arterial PO2 did not return to control
levels before the start of the next hypoxia exposure (2). Thus
our IH-C protocol produces blood-gas changes more similar to
those reported in clinical studies of OSA (6) and may be more
relevant to the human condition where sleep apnea is correlated
with an increased incidence of hypertension (28).

In the earlier studies by Fletcher and coworkers (12), blood
pressure was recorded at day 0 and after 35 days exposure to
either 120 episodes/h of hypoxia only (inspired O2, 2.5%) or
hypoxia supplemented with CO2 (8% or 14% compared with
5% in our studies). These earlier studies did not measure blood
pressure at earlier time points in the IH-C groups but did
observe that the acute increase in blood pressure and sympa
thetic nerve activity was greater with IH-C than with IH
exposure (22). It is therefore interesting to speculate that added
CO2 may accelerate humoral and neural responses to IH,
similar to observations that hypercapnic hypoxia in humans
produces a more profound activation of the sympathetic nervous
system than IH only (23).

Similar to a previous study using the IH-C protocol (20),
plasma ET-1 levels in untreated IH-C rats were significantly
higher than in Sham rats. Furthermore, the increase in circu
lating ET-1 and in blood pressure during IH-C exposure was
prevented by simultaneous administration of the superoxide
dismutase mimetic, Tempol. In contrast, Tempol had no effect
on MAP or plasma ET-1 in Sham rats. Therefore Tempol
appears to counteract an IH-C-exposure effect, perhaps by
eliminating ROS-stimulated ET-1 production (5, 19), as sug
gested by the measures of vascular ROS. Thus our data suggest
that in vivo oxidative stress in IH-C rats, and potentially OSA
patients, contributes to the development of hypertension by
stimulating ET-1 synthesis.

Three separate indicators measured increased ROS pro
duction in small mesenteric arteries from IH-C rats com
pared with arteries from sham-operated rats. Because ET-1
can activate NADPH oxidase (29), it is possible that ele
vated ET-1 stimulated ROS production in the vascular wall
of IH-C rats. This would suggest the increased ROS results
from ET-1 receptor activation. However, it is unclear how a
feed-forward system might operate in vivo with ET-1 pro
duction both stimulated by superoxide and increasing su
peroxide production. Furthermore, the observation that the O2
scavenger, Tempol, prevented both increases in circu
lating ET-1 and IH-C-induced increases in MAP makes it
more likely that the increase in ROS is upstream of ET-1
production rather than downstream.

Previous studies of ET-1-dependent hypertension have dem
onstrated that Tempol lowers blood pressure and ROS produc
tion (35), that it lowers vascular ROS production without
decreasing blood pressure (10), or that it decreases blood
pressure independent of its effects on superoxide production
(40). For example, Xu et al. (39) suggested that Tempol
decreases MAP through direct inhibition of sympathetic neu
rons in the cardiovascular system. This may have contributed
to the decrease in the MAP we observed and would be in
agreement with previous studies demonstrating that blocking
the sympathetic nervous system inhibits the development of

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Fig. 5. Plasma concentration of endothelin (ET-1) in blood samples taken
from rats exposed to 14 days of either Sham or IH-C cycling and drinking
either tap water or water containing Tempol (1 mmol/l). [ET-1] was signifi
cantly greater in plasma from IH-C rats compared with plasma from sham
operated rats and plasma from Tempol-treated IH-C rats (P < 0.05). *Signif
dicant difference from Sham; #Significant difference from vehicle within group.

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REACTIVE OXYGEN SPECIES IN INTERMITTENT HYPOXIA HYPERTENSION

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hypertension in other protocols of IH-C exposure (22). Because the in vivo treatment with Tempol lowered both the vascular ROS generation and the increases in ET-1 and MAP, the data also support the hypothesis that ROS act at the level of the vascular wall to stimulate ET-1 production, which in turn elevates MAP. Therefore, additional studies with ET-1 receptor antagonists are needed to establish which is the sequence of events.

In conclusion, the cellular mechanisms connecting sleep apnea to ROS generation and subsequent hypertension are not entirely clear. The current studies provide a novel potential link, suggesting repetitive exposures to brief periods of eucapnic hypoxia lead to increased vascular ROS generation and subsequent hypertension are not events.

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References


