A new rodent model for obstructive sleep apnea: effects on ATP-mediated dilations in cerebral arteries

Randy F. Crossland,1,3 David J. Durgan,1 Eric E. Lloyd,1 Sharon C. Phillips,1 Anilkumar K. Reddy,2 Sean P. Marrelli,1,3 and Robert M. Bryan, Jr.1,2,3

1Department of Anesthesiology, Baylor College of Medicine, Houston, Texas; 2Department of Medicine (Cardiovascular Sciences), Baylor College of Medicine, Houston, Texas; and 3Department of Molecular Physiology and Biophysics (Graduate Program in Cardiovascular Sciences), Baylor College of Medicine, Houston, Texas

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Obstructive sleep apnea (OSA) is characterized by complete (apnea) or partial (hypopnea) obstruction of the airway during sleep with an ongoing effort to breathe (15, 18, 43). In adults, OSA is most commonly caused by a collapse of the upper airway resulting from decreased tone in the pharyngeal dilator muscles during sleep. Risk factors for OSA include obesity, aging, sex, and craniofacial anatomy (43). While OSA appears to be more prominent in males, it does commonly occur in females. The incidence of clinically significant OSA has been estimated between 5 and 25% of the adult population in the Western world (18).

OSA is strongly associated with metabolic and cardiovascular diseases (15, 18, 43); however, the exact nature of this relationship has been difficult to establish given that OSA is most often accompanied by confounding comorbidities (such as obesity). One important means to separate OSA from the confounding comorbidities present in humans has been the use of animal models (14, 21). The most commonly used model involves exposing rodents to chronic intermittent hypoxia (generally 3–10% O2) during the sleep cycle. Chronic intermittent hypoxia models mimic the hypoxia and reoxygenation characteristics of OSA. However, apnea, which occurs in human OSA, is not present in intermittent hypoxia models. Apnea and the accompanying negative intrathoracic pressure that occurs when breathing effort continues against a closed airway have unique pathophysiological consequences (43). The first goal of this study was to develop a chronic model of OSA in rats that included apneas during the sleep cycle. By incorporating apnea, the model should more closely mimic OSA as it occurs in humans.

A second goal of this study was to determine the effects of OSA on the cerebrovascular wall. The rationale behind this goal was 1) human studies demonstrate that OSA decreases cerebral blood flow, produces autoregulatory dysfunction, and diminishes cerebrovascular responses to hypoxia and hypercapnia; 2) OSA is an independent risk factor for stroke, a cardiovascular disease; 3) stroke patients with OSA have poorer prognosis and increased risk of stroke recurrence compared with non-OSA patients; and 4) a vascular component with diminished cognitive function is suggested in patients with dementia and OSA (18). Although the above implicates a major effect by OSA on the cerebrovascular wall, only two studies to date have addressed this issue using intermittent hypoxia in rodents (10, 37).

One important component of cerebrovascular control is the regulation of arterial diameter by endothelial-dependent processes. In rat cerebral arteries, endothelium-dependent dilations are controlled by two processes: 1) nitric oxide (NO) and 2) endothelium-dependent hyperpolarization (EDH) (2, 8, 28, 49). For example, ATP, a naturally occurring agonist in plasma, dilates cerebral arteries through generation of NO at lower concentrations and EDH at higher concentrations (2, 8, 28, 49). EDH in cerebral arteries involves hyperpolarization of endothelial cells by activation of intermediate, and possibly, small conductance calcium-activated potassium channels (8). The hyperpolarization spreads from the endothelium to the vascular smooth muscle through myoendothelial gap junctions where it closes voltage-operated calcium channels in the smooth muscle to elicit dilation (8, 41). Endothelial dilator function in cerebral arteries and arterioles is affected by a number of pathological conditions (20). Of interest, pathological conditions, including traumatic brain injury, ischemia/

Address for reprint requests and other correspondence: R. M. Bryan, Jr., Dept. of Anesthesiology, Rm. 434D, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030 USA (e-mail: rbryan@bcm.edu).

reperfusion, or head-down tilt, a model for microgravity in rodents, attenuated dilations through endothelium-derived NO but enhanced the EDH dilator component (8, 13, 27, 34, 38). Therefore, we have used our new model of OSA, which incorporates apnea, to test the hypothesis that OSA attenuates dilation through endothelium-derived NO and enhances dilations through EDH.

MATERIALS AND METHODS

All protocols were approved by the Institutional Animal Care and Use Committee at Baylor College of Medicine, Houston, TX. Animals were housed in a satellite facility with a 12:12-h light (8 AM–8 PM)-dark (8 PM–8 AM) cycle. Male Long-Evans rats 8–9 wk old at the time of surgery were used for the study. Apneic events were achieved by remotely inflating a balloon or obstruction device, which was implanted into the trachea, in freely ranging rats.

Endotracheal obstruction device and implantation. Obstruction devices were constructed from 5-cm Renasil silicone tubing (SIL037; Braintree Scientific). The silicone tubing was closed at one end with 5–0 suture silk. The open end of the silicone tubing was joined to 51 cm of PE-50 tubing (BB31695-PE/3; Scientific Commodities, Lake Havasu City, Arizona) (Fig. 1A). A 5-mm segment, near the sealed end of the silicone tubing, was gently stretched 10 times to twice its original length to weaken the wall. Upon increasing the pressure within the silicone tubing, the stretched segment would inflate to ~3 times its original diameter (Fig. 1A).

Animals were anesthetized with CCM DEAIII-Rodent Cocktail (ketamine, 37.5 mg/ml; xylazine, 1.9 mg/ml; acepromazine, 0.7 mg/ml; 1 µl/g body wt). A 2-cm midline incision was made just above the sternum. Connective tissue and the sternohyoid muscles were gently spread to visualize the trachea. Two small holes were made in the trachea using a hypodermic needle. The first hole was two cartilage rings below the thyroid, and the second hole was located three cartilage rings caudal to the first hole. The obstruction device was inserted into the first hole and advanced until it could be pulled out of the second hole (Fig. 1B). A piece of silk suture attached to the closed end of the obstruction device served to pull the obstruction device into the first hole in the trachea and out of the second hole. Upon inflation, the obstruction device completely blocked the airway (Fig. 1B). The free end of the PE-50 tubing connected to the silicone obstruction device was tunneled under the skin to the midline between the shoulder blades where it exited.

The sternohyoid muscles and skin were closed by suture. The PE-50 tubing exiting the back of the neck was secured to the skin using a suture. The PE-50 tubing was passed through an 18” metal tether using a harness (SAH-18; Strategic Applications) to secure the tether to the rat at one end and to a swivel (QCS-S; Strategic Applications) at the other end (Fig. 1C).

Beginning the day of surgery, the animals were treated for 3 days with an analgesic (5 mg/kg ketoprofen; Fort Dodge Animal Health, Fort Dodge, IA) and an antibiotic (5 mg/kg Baytril; Bayer HealthCare LLC, Animal Health Division, Shawnee Mission, KS). Animals were allowed to recover for 1 wk before apneas were initiated.

Inflation of obstruction device in freely ranging rats. Animals were housed in standard rat cages with lids modified to accommodate the swivel/harness system used to tether the rats. Apneas were controlled by a computer connected to a syringe pump (BS8000; Braintree Scientific), and a pressure feedback system (Fig. 1C). Upon reaching the inflation pressure for each balloon, the pump paused holding the obstruction device inflated for 10 s before withdrawing to deflate the obstruction device. We could determine whether inflation was occurring by observing the chest movement of each rat. A noticeable and exaggerated chest movement occurred during apnea when the rats attempted to breathe against a closed airway. This method of determining apnea was confirmed using pulse oximetry, blood gas analysis, and airway pressure measurements. In all cases, we were able to determine with certainty whether apneas were occurring. Rats were observed at the beginning of apneas every day, and the balloon inflation pressure was adjusted as needed to ensure that apneas were occurring. As a consequence of the extensive monitoring, we can state with certainty that the rats underwent episodes of apnea throughout the 4-wk period of the study.

When the balloon and the hydraulic system were intact, pressure in the system remained constant for the 10-s duration of the apnea (balloon inflation). The pressure as measured by the pressure feedback system was constantly displayed on a video screen and monitored at regular intervals throughout the sleep cycle. If the pressure did not maintain a plateau during the apnea, the source of the leak was located (swivel or tube connections) and repaired, or if the leak were in the balloon, the animal was removed from the study.

The rats were exposed to 10 s of apnea at a rate of 30 apneas/h for 8 h a day during the sleep cycle (9 AM–5 PM) for 4 wk. Each apnea randomly occurred within a 2-min period of time. This number of apneas per hour represents a moderate level of OSA in humans (43). Sham rats, which were instrumented as described above but did not undergo apneas, served as controls. Rats were instrumented and run in groups of eight with four rats randomly assigned to the sham group (without apneas) and four rats assigned to the OSA group (apneas). An eight-channel video surveillance system allowed continuous monitoring of individual rats (SY70308; Supercircuits, Austin, TX).

Airway pressure study. In a subset of animals, airway pressures were studied before and after implantation of the obstruction device. Rats were anesthetized with 1.5% isoflurane, and the trachea was

Fig. 1. A: obstruction device consisting of Renasil silicone tubing joined to polyethylene (PE)-50 tubing. Picture insets show obstruction device deflated (left) and inflated (right). B: diagram of the obstruction device in the rat trachea. C: diagram of rat and system controlling apneas. The system consists of a computer to initiate apneas, a programmable syringe infusion/withdrawal pump, and a pressure transducer.
Innovative Methodology

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Exposure as described above. The tip of a saline-filled catheter, constructed of 20 cm of PE-50 with a tip made of 2 cm PE-10, was inserted between the site for implantation of the obstruction device and the lung. Pressures were monitored in the trachea before and after implantation of the obstruction device and after inflation of the obstruction device.

Blood gas analysis, plasma analysis, and blood pressure monitoring. In a subset of rats, a chronic indwelling catheter was placed in the abdominal aorta as previously described (5, 7). Briefly, a catheter was constructed of a 63-cm-long piece of Renapulse tubing (RPT-037; Braintree Scientific) connected to a 5-cm-long piece of microurtheuma tubing (BB520-25; Scientific Commodities). The catheter was filled with a 10 U/ml heparin solution. A 4-cm-long midline incision was made caudally, starting 1 cm below the sternum. Viscera were removed from the abdominal cavity to visualize the abdominal aorta. A short segment of the aorta proximal to the iliac artery bifurcation was briefly occluded, and the microurtheuma tubing was inserted through a hole in the aorta made by a 23-gauge needle. A drop of tissue cement was applied to the insertion site. The viscosa were replaced, and the abdominal muscles and skin were closed. The catheter tubing was passed through the back and tunneled under the skin to the same site where the tubing for the obstruction device exited. The catheter was held in place by suturing it to the psoas muscle. In these experiments, a dual-channel swivel (SVLRF2F22; Kent Scientific, Torrington, CT) was used to connect both the arterial catheter to a pressure transducer through one channel and the obstruction device tubing to the syringe pump through the other channel. Rats were given 1 wk to recover from surgery before initiation of apneas.

Blood pressure was monitored for up to 4 wk in OSA and sham rats. The transducers were interfaced with a PowerLab data acquisition system with LabChart 7 (AD Instruments, Colorado Springs, CO).

Blood samples (200 μl) were taken before and at the end of apnea to measure blood gas and pH using IRMA TruPoint Blood Gas Analyzer (International Technidyne, Edison, NJ). Hemoglobin oxygen saturations were calculated from blood PO2 and pH values using a saturation curve for rats (12).

In some rats, blood samples (1 ml) were collected in EDTA tubes after 4 wk of apnea or after an equivalent time in sham control rats. Plasma was isolated by centrifuging the samples at 1,000 g for 15 min at 4°C. Plasma TNF-α levels were determined by ELISA (RTA00, R&D Systems, Minneapolis MN). Plasma lipid panel and plasma lipid panel and plasma were determined in nonfasted rats by the BCM Center for Comparative Medicine Pathology Core.

Oxidative stress was quantified in protein lysates after derivatizing carbonyl groups, a marker of protein oxidation, to 2,4-dinitrophenylhydrazine with 2,4-dinitrophenylhydrazine using Oxyblot protein oxidation kit (S7150; EMD Millipore, Billerica MA). Protein was isolated from heart, lung, liver, kidney, and aorta of both sham and OSA rats. The level of derivatized carbonyl groups was compared with a loading control, GADPH determined by probing with an anti-GADPH antibody (cat. no. CB1001; Calbiochem, San Diego, CA). Blots were analyzed and quantitated with ImageJ software (National Institutes of Health, Bethesda, MD).

Left ventricular echocardiography and Doppler ultrasonography of blood flow velocities. After 4 wk of apnea in the OSA group and an equivalent period of time in the sham control group, rats were anesthetized with 1.5% isoflurane. Morphology and function of left ventricle were determined by echocardiography using a VisualSonics VeVo Imaging system (VisualSonics, Toronto, Canada) with a 25-MHz probe (44, 46). Motion (M)-mode images at the level of the papillary muscles were captured and analyzed offline. Blood flow velocities through the mitral valve, aorta, and carotid arteries were measured using Doppler ultrasonography with a 2-mm diameter 20-MHz probe (30, 31).

Isolated middle cerebral arteries. Four weeks after initiation of apneas for the OSA group or the equivalent time for sham-control group, rats were anesthetized with isoflurane, and the brain was rapidly removed and placed in cold Krebs buffer. Left and right middle cerebral arteries (MCAs) were carefully harvested and placed in a vessel chamber for the study of pressurized and perfused MCAs (6, 9, 48). The vessel chamber was filled with Krebs buffer consisting of (in mM) 119 NaCl, 4.7 KCl, 1 MgSO4, 1.2 KH2PO4, 0.026 EDTA, 1.6 CaCl2, 5.5 glucose, and 25 NaHCO3. The buffer was gassed with 20% O2, 5% CO2, balanced with N2 to obtain a pH of 7.4. Both ends of each MCA were mounted on glass micropipettes and secured with 12–20-nl suture. The MCAs were pressurized to 85 mmHg by raising buffer-filled reservoirs connected to the glass micropipettes above the MCA. Care was taken to ensure that the mounted vessel segments did not have side branches or leaks. After 30 min of pressurization, luminal flow (150 μl/min) was initiated by adjusting the heights of the reservoirs to create a pressure gradient across the vessel system. MCAs were allowed an additional 30 min to develop spontaneous tone before the experimental protocol began.

Endothelium-mediated dilations were studied by applying ATP to the reservoir perfusing the lumen of each MCA. In this manner, ATP has preferential access to the P2Y2 receptors expressed on the luminal surface of endothelium (48). ATP, which is released from a number of cell types, including red blood cells and endothelium, is a naturally occurring agonist in the plasma (19). ATP was applied at three concentrations (10−7 M, 10−6 M, and 10−5 M) with a 5-min wash between doses. In some studies, 100 μM nitro-L-arginine methyl ester (L-NAME) was used to inhibit NO synthase; 10 μM indomethacin was used to inhibit cyclooxygenase. The inhibitors were administered luminally and abuminally 45 min before initiating the ATP dose-response dilation. Only one ATP dose-response dilation was conducted for a single MCA. In some studies, NO synthase was inhibited with L-NAME, and the NO donor, methylamine hexamethylene methyamine NONOate (MAHMA-NOnoate (MAHMA-NOnoate, 10−8 M–10−3 M) was administered abuminally to determine the sensitivity of the vascular smooth muscle cell to NO.

At the end of every study, MCAs were bathed in Ca2+-free Krebs buffer to determine maximal diameter. Vessel studies were digitally recorded by a CCD camera connected to a DVR. Data were analyzed offline using edge detection software to follow changes in vessel outer wall diameter. Responses to ATP were normalized as a percentage of maximal diameter as defined by % maximal diameter = 100 × (Dmax − Dbase)/(Dmax − Dbase), where (Dmax − Dbase) was measured immediately before the addition of the first dose of ATP, and Dbase was maximum diameter of the MCA in Ca2+-free buffer).

Statistics. Data are expressed as means ± SE. For comparing a single measure in two groups, a Student’s t-test was used. For analyzing the dose-response dilation, a two-way repeated-measures ANOVA was used followed by a Holm-Sidak post hoc test for individual comparisons when appropriate.

Drugs and reagents. All reagents and drugs were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified. Indomethacin dissolved in a solution of Na2CO3 and distilled water.

RESULTS

Rat model of OSA. Table 1 shows characteristics of OSA rats after 4 wk of apnea and the equivalent time in sham-operated rats. There were no significant differences in weight gain, final body weight, or the ratios of the heart, lung, and liver to the length of the tibia (n = 7 each group). The tibia length was used to normalize these values since it has less variation than body weight for a given average. Furthermore, there were no differences in water or food intake throughout the surgery recovery period (first week) and the following 4 wk in the OSA

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group and the sham control rats ($n = 5$ and $8$, respectively, data not shown). Throughout the experimental protocol, no audible congestion or wheezing was evident in either shams or OSA rats. Post mortem examination of tracheas with implanted obstruction devices revealed no visual signs of mucus accumulation, tissue damage, or inflammation. Thus, we conclude that the implants were well tolerated by all animals.

Rats were observed throughout the 24-h cycle by a video surveillance system. During the sleep cycle, rats were generally in a “resting state” (sleeping posture). A few seconds after apnea was initiated, we observed enhanced chest movements, resulting from a continued and enhanced breathing effort against a closed airway. After termination of the apnea or during the last few seconds of the apnea, rats were aroused as noted by head raising or standing. This behavior was suggestive of arousal from sleep, a characteristic of OSA in humans. After the arousal behavior, rats immediately returned to the “resting state” until the next apneic episode occurred (Supplemental Video S1). Because we did not have EEG recordings, we could not conclusively determine whether rats were asleep or in what stage of sleep when individual apneas occurred. On occasions, a period of apnea would occur when the rat was clearly awake; however, even when awake, the apneas did not produce behaviors more excessive than mild agitation.

Figure 2A shows airway pressure tracings before, during, and after an apneic episode in isoflurane-anesthetized rats. In Fig. 2A, left, the apnea was initiated at end inspiration [when the lungs were inflated], and in Fig. 2A, right, the apnea was initiated at end expiration [when the lungs were deflated]. Note the progression of the minimum airway pressure with each attempt to inhale against the closed airway. Also note that the minimum airway pressure was more negative when apneas were initiated at end expiration. Fig. 2B shows summary data for maximum and minimum airway pressures during the respiratory cycle before implantation of the obstruction device, after implantation, and after an apneic episode in isoflurane-anesthetized rats. In both groups, the minimum airway pressure was more negative when apneas were initiated at end expiration ($P = 0.001$), $P_{O_2}$ from $122 \pm 3$ to $67 \pm 3$ ($P = 0.001$), $P_{CO_2}$ from $43 \pm 1$ to $51 \pm 1$ ($P = 0.003$), and calculated $O_2$ saturation of hemoglobin decreased $12\%$ from $94 \pm 0$ to $82 \pm 1$ ($P = 0.001$) ($n = 5$ for each).

Table 1. Comparison of OSA rats and sham-control rats after 28 days of apnea or device implantation without inflation, respectively

<table>
<thead>
<tr>
<th></th>
<th>Sham ($n = 7$)</th>
<th>OSA ($n = 7$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final body weight, g</td>
<td>$400 \pm 21$</td>
<td>$410 \pm 23$</td>
</tr>
<tr>
<td>Weight gain, g</td>
<td>$100 \pm 14$</td>
<td>$94 \pm 8$</td>
</tr>
<tr>
<td>Tibia length, mm</td>
<td>$15.0 \pm 0.1$</td>
<td>$14.5 \pm 0.2$</td>
</tr>
<tr>
<td>Heart weight: tibia length, g/mm</td>
<td>$0.08 \pm 0.00$</td>
<td>$0.07 \pm 0.00$</td>
</tr>
<tr>
<td>Lung weight: tibia length, g/mm</td>
<td>$0.1 \pm 0.0$</td>
<td>$0.1 \pm 0.0$</td>
</tr>
<tr>
<td>Liver weight: tibia length, g/mm</td>
<td>$1.2 \pm 0.1$</td>
<td>$1.2 \pm 0.1$</td>
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Values are expressed as means $\pm$ SE. OSA, obstructive sleep apnea.

Fig. 2. A: airway pressure before, during, and after an episode of apnea when the apnea occurred at end inspiration (lungs inflated, left) and end expiration (lungs deflated, right). B: summary data for maximum and minimum airway pressures before implantation of the obstruction device, after implantation, and during apneas beginning on end inspiration or end expiration. *$P = 0.01$ compared with maximum of all other groups; **$P < 0.01$ compared with minimum for all other groups; ***$P < 0.001$ compared with minimum for all other groups.

Fig. 3. Arterial pH, $P_{O_2}$, $P_{CO_2}$, and calculated $O_2$ saturation of hemoglobin at baseline and at the end of a 10-s period of apnea. *$P \leq 0.003$. 

(7)
Effects of apnea on blood pressure and cardiac function/morphology. Using a chronic indwelling arterial catheter, we monitored the mean arterial blood pressure to determine the acute and chronic effects of OSA on blood pressure. Acute apnea elicited several different types of blood pressure responses, as is commonly seen in patients. Figure 4A shows three different responses observed during apnea: 1) the MABP steadily increased during the apnea, transiently dipped at the end of the episode, and then spiked (Fig. 4A, top); 2) the MABP transiently decreased at the end of the episode (Fig. 4A, middle); 3) the MABP changed very little during and after the apnea (Fig. 4A, bottom). Because we did not determine sleep stages during apnea exposure, we cannot discount that the difference in the MABP response was due to the sleep state of the animal. Of interest, each of these types of responses could occur in the same animal. The acute blood pressure response to apnea did not appear to correlate with body position, number of days of apnea, or the time during the sleep cycle.

Figure 4B shows mean arterial blood pressure during the sleep phase (8 AM–8 PM, top) and awake phase (8 PM–8 AM, bottom) in sham or OSA rats at 7-day intervals during the study period. There were no significant differences in mean arterial blood pressure between groups for any of the days. Thus, apneas did not produce hypertension in this model of OSA over 4 wk.

To assess morphology and function of the left ventricle, we examined OSA rats exposed to 4 wk of apnea and sham control rats after an equivalent period of time using echocardiography and Doppler ultrasound. Inner volume, posterior wall thickness, and anterior wall thickness were similar between groups during systole and diastole (n = 6 and 8 for OSA and sham controls, respectively, data not shown). Furthermore, apneas did not produce significant changes in stroke volume, ejection fraction, or cardiac output (data not shown). Doppler ultrasound of flow velocities in the mitral valve, aorta, and carotid artery were similar between OSA and sham control rats (n = 5 and 7, respectively, data not shown). Repeated episodes of apnea (10 s each) at a rate of 30 apneas/h during 8 h of the sleep cycle for 4 wk did not produce any significant alteration in blood pressure, left ventricular morphology and function, or peripheral vessel flow velocities.

Effects of apneas on inflammation, oxidative stress, and plasma lipids and glucose. Plasma TNF-α was similar in OSA (n = 5) and sham control rats (n = 5), indicating that apneas for 4 wk did not produce an enhanced inflammatory state (Fig. 5A). Figure 5B shows levels of carbonyl groups on protein, a marker of oxidative stress. The levels of carbonyl groups were not different between OSA (n = 5) and sham control rats (n = 5) in heart, kidney, liver, or aorta. Unexpectedly, the level of carbonyl groups decreased in the lung of OSA rats compared with sham control rats (P = 0.007). We cannot explain this paradoxical decrease in carbonyl levels in lung; however, it could involve enhanced antioxidant defense in lung in response to oxidative stress (29, 39). There were no differences in plasma glucose, triglycerides, total cholesterol, high-density lipoproteins, low-density lipoproteins, or very low-density lipoproteins between the OSA and sham control rats, suggesting an absence of metabolic alterations (n = 5 for each group, data not shown).

Effects of apneas on middle cerebral artery dilations. Maximum diameters for rat MCAs were 304 ± 7 (n = 10) and 305 ± 9 (n = 7) for sham control and OSA rats, respectively (P = 0.91). After developing spontaneous tone, diameters of MCA from the two groups were 226 ± 6 and 219 ± 10, respectively (P = 0.48). Figure 6A shows dose-dependent dilations in response to ATP in MCA. Note that the dilations are significantly suppressed in vessels from OSA rats (P = 0.04 for a group effect; n = 10 and 7 for sham control and OSA groups, respectively). Dilations at 10⁻⁶ and 10⁻⁵ M ATP in OSA arteries were decreased by 38% (P = 0.003) and 17% (P = 0.038) of the corresponding dilation in the sham MCAs, respectively.

Since stimulation of endothelial P2Y₂ receptors by ATP dilates cerebral arteries through endothelial NO production and by EDH (8, 49), further studies were conducted to determine which component of the dilation was affected by the 4 wk of apnea. The effects of blocking NO synthase with L-NAME (10⁻⁴ M) alone or in combination with indomethacin (10⁻⁵ M), an inhibitor of COX, on the dilator response to ATP are shown in Fig. 6, B and C, respectively. Inhibition of NO synthase decreased the dilatory response at 10⁻⁷ M and 10⁻⁶ M ATP in MCAs from both the sham control and OSA rats.
(compare responses in Fig. 6B to 6A). However, the dilations in the presence of L-NAME were not different between sham control (n = 4) and OSA rats (n = 4, group effect; P = 0.22). The addition of indomethacin to L-NAME had no further effect on inhibiting the dilation than L-NAME alone (sham control n = 9; OSA n = 7; group effect P = 0.13). Thus, the dilations to ATP did not have a component involving COX metabolites in either group. The EDH component of the dilation (i.e., the dilation remaining after inhibition of NO synthase) was not affected by 4 wk of apneas compared with the sham control rats.

Figure 7 shows that dilations to the NO donor, MAHMA-NONOate, were similar in MCAs from sham control and OSA rats (n = 6 for each group). Thus, decreased smooth muscle sensitivity to NO cannot account for suppressed dilations to ATP. Assuming an additive effect of NO and EDH in the ATP-mediated dilations, the NO component must have been suppressed since the total dilation was suppressed, but the EDH component was not.

**DISCUSSION**

The purpose of these studies was to 1) develop a chronic model of OSA in rats that incorporates apnea to more closely recapitulate OSA as it occurs in humans and 2) to determine whether endothelial function is altered in cerebral arteries from rats undergoing 4 wk of apneas. To this end, we successfully developed the model in rats and describe the physiological effects after 4 wk of apneas. The rat model, consisting of 30 apneas/h, is considered moderate OSA in humans. We conclude that this “moderate” level of OSA for 4 wk was not sufficiently severe to produce overt systemic physiological changes, such as hypertension, in young healthy rats. Next, we demonstrated that endothelial dilator function to ATP was attenuated in isolated MCAs as a result of reduced NO bioavailability after 4 wk of apneas. Thus, the cerebral circulation appears to be very sensitive to the pathological effects of repeated apneas and shows dysfunction before overt systemic changes occur.

**OSA model.** Animal models have been an important tool for studying OSA and other types of sleep-disordered breathing. One clear advantage is that animal models can be studied without the confounding comorbidities commonly occurring with humans suffering from OSA. A number of models in larger animals and rodents have involved obstruction of the airway (1, 11, 23, 40) (see Refs. 14 and 21 for reviews of animal models); however, these studies focused on the acute aspects of OSA and/or did not describe the chronic effects of the airway obstruction. More chronic models in cats and rats were developed, but both models require restraint of the animal (22, 35). In the 1990s, one group of researchers developed a sophisticated model in dogs that was able to induce apneas (up to 60 apneic events per hour) for 3 mo (4, 32). To our knowledge, this group has published the only previous studies in which obstructions to the airway were utilized over longer time periods in nonrestrained animals. Although the studies from this group provided important information regarding the pathophysiology of OSA, publications with this model have not appeared in the literature in more than 10 years.

The model most often used has been chronic intermittent hypoxia (CIH) in which the ambient O2 is reduced to 3–10%
from 9–60 times/h during the sleep cycle of rats and mice (14, 16, 21, 25, 26). This CIH model and variations of it have parallels with OSA in the human and have provided needed insight into the pathological mechanisms of OSA. Although CIH has been and will continue to be an important animal model, we wanted to develop a chronic rodent model that used apnea and encompassed more of the characteristics of human OSA.

Model limitations. Ideally, an animal model for OSA should include 1) apnea, 2) progressive hypoxia with reoxygenation, 3) progressive hypercapnia, 4) arousals from sleep, 5) sleep deprivation, and 6) negative intrathoracic pressures. Our model incorporates the above (some limitation discussed below) with the possible exception of sleep deprivation (Table 2). Since the rats in our model had 240 episodes of apnea during the sleep cycle (30/h × 8 h) and the rats are clearly aroused, it would first seem that sleep deprivation should occur. However, it is possible that lost sleep occurring during the sleep cycle was made up during the awake cycle. Ongoing studies measuring activity of the rats over the course of weeks should shed light on this issue.

Other limitations of our model include the timing of the apneas. Although apneas occurred during the sleep cycle, apneas did not necessarily occur when the rat was asleep. We often observe rats in a curled “sleep-like” or resting postures with the onset of apnea (see Supplemental Video S1); however, without EEG recording, we cannot definitively state whether the animals were asleep or, if asleep, the specific sleep stage. Without knowledge of the sleep state, we cannot determine whether the arousal-like reactions occurring at the end of the apneas were truly an arousal from sleep. Issues with the onset of apnea as it relates to sleep or the sleep stage can be resolved without knowledge of the sleep state, we cannot determine whether the arousal-like reactions occurring at the end of the apneas were truly an arousal from sleep. Issues with the onset of apnea as it relates to sleep or the sleep stage can be resolved

Outcomes of 4 wk of apneas. After 4 wk of apnea, we did not observe overt changes in the physiological state; that is, OSA rats and sham control rats had no statistically significant differences in mean arterial blood pressure, body size, inflammatory state (TNF-α), oxidative stress (as determined by changes in protein carbonyl groups), plasma lipids, plasma glucose, left ventricular function or morphology, and blood flow velocities through the mitral valve, carotid artery, and aorta.

We were initially surprised that we did not observe hypertension in our model after 4 wk of apneas. Rats undergoing intermittent hypoxia often develop hypertension after a few weeks (14). In addition, Brooks et al. (4) reported dogs became hypertensive over 1 to 3 mo when undergoing apneas during sleep. We speculate that hypertension did not develop in our model as a result of a more moderate level of insult. O2 desaturations with CIH models often model severe OSA having O2 desaturations up to 30% (14, 16, 21, 36). In comparison, the rats in our study desaturated by 12%. We choose this level of desaturation to be consistent with those commonly observed in OSA patients (3, 24, 42, 43). Although the level of O2 desaturation was not reported in the study by Brooks et al. (4), the frequency of apneas was two-fold greater (after progressively increasing the frequency over 1 wk) than those used in the present study. We speculate that the development of hypertension is dependent on the severity of the insult in the model. In models in which O2 desaturations are more pronounced or the frequency of the insult is increased, the development of hypertension (and other cardiovascular pathologies) should occur at earlier times than models that are more moderate.

We believe that our model may provide interesting insight into OSA as it translates to the human condition. The young healthy male rats in our study tolerated “moderate” levels of OSA for 4 wk without overt signs of systemic pathology. We raise the idea that young healthy humans may tolerate “moderate” levels of OSA for extended periods without overt clinical symptoms. Given that OSA is most often associated with an existing comorbid condition (i.e., obesity), we speculate that an interaction between OSA and the comorbid conditions rapidly accelerates the pathology to the point of overt clinical symptoms.

Studies on middle cerebral arteries. Despite the lack of hypertension or other systemic changes, we did find that MCAs from rats after 4 wk of apneas had an attenuated dilator response to luminally applied ATP. Luminally applied ATP stimulates P2Y2 receptors to dilate MCAs through NO and EDH (8, 47, 49) (see introduction). Of interest, following many

Table 2. Desired conditions for animal OSA model

<table>
<thead>
<tr>
<th>Desired Condition for Modeling OSA</th>
<th>Presence in Most Chronic Models of OSA</th>
<th>Our Model</th>
<th>Comment/Limitation for our Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apnea</td>
<td>Not present</td>
<td>Yes</td>
<td>1) Apneas occurred during the sleep cycle. Rats were sometimes awake when an apnea was initiated. 2) Apneas occurred during any part of the respiratory cycle. With OSA in humans, airway collapse occurs at the beginning of inspiration when the lungs are deflated.</td>
</tr>
<tr>
<td>Progressive hypoxia with reoxygenation</td>
<td>Yes</td>
<td>Yes</td>
<td>An evoked reaction, appearing to be an arousal, occurred when the rat was in a sleep-like posture. However, the sleep wake-state is not known at the time apnea occurs.</td>
</tr>
<tr>
<td>Progressive hypercapnia</td>
<td>Rarely</td>
<td>Yes</td>
<td>Sleep could be made up during awake cycle.</td>
</tr>
<tr>
<td>Arousal</td>
<td>Sometimes</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Sleep deprivation</td>
<td>Not known</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Negative intrathoracic pressures</td>
<td>Not known</td>
<td>Yes</td>
<td></td>
</tr>
</tbody>
</table>
pathological conditions, the NO component of the dilation is attenuated, and the EDH component is enhanced (8, 13, 33, 34, 38). It is thought that this upregulation of EDH can function to offset the diminished NO bioavailability seen in pathological conditions.

On the basis of these previous observations regarding NO and EDH during pathological conditions, we initially hypothesized that OSA 1) attenuates dilation by reducing endothelium-derived NO and 2) enhances dilations through EDH. In this study, we demonstrate that our hypothesis regarding attenuated NO dilations is valid, while our hypothesis regarding enhanced EDH dilations is not valid. Dilations to ATP were attenuated in MCAs from rats after 4 wk of apnea compared with sham control rats, while the EDH component (determined after blocking NO synthase alone or in combination with inhibition of cyclooxygenase) was not affected. It follows that the NO component of the dilation must have been attenuated. Of note, the dilation to the NO donor, MAHMA NONOate was similar in MCAs from sham and OSA rats, indicating that the dilator response by vascular smooth muscle was unaltered. Given the above observations, we conclude that 4 wk of apneas alters endothelial function by limiting NO bioavailability in cerebral arteries. In addition, to the attenuated endothelium-mediated dilations, the constrictor response to endothelin-1 was significantly enhanced in our model of OSA (17). The enhanced constrictor response was the result of an increased activity of the endothelin receptor type B (17).

Our results involving attenuated NO bioavailability can be compared with previous studies using the CIH model. The increase in cortical perfusion with the application of ACh, an agonist that dilates arteries and arterioles by activating endothelial NO synthase, was attenuated by 41% in mice after 35 days of CIH (10) and decreased dilations of isolated MCAs in rats by 75% after only 2 wk of CIH (37). Capone et al. (10) also demonstrated neovascular coupling was significantly affected by CIH. The fact that changes in cerebral arterial function was observed in our model without any overt systemic changes is indicative of the sensitivity of the cerebral circulation to pathological states (20).

One common cause of attenuated endothelium-mediated dilations by NO involves an increased production of reactive oxygen species (ROS), often in the form of superoxide generated from the enzymatic reaction catalyzed by NADPH oxidase (10, 20). ROS can react directly with NO to reduce its availability, while also interfering with the ability of NO synthase to generate NO (20). Interestingly, we did not find any signs of oxidative stress in peripheral tissues, including heart, lung, aorta, or kidney. Unfortunately, we were unable to obtain sufficient cerebrovascular tissue to determine whether oxidative stress increased in our OSA model. However, the cerebral circulation is known to be more sensitive to pathological conditions and has greater concentrations of NADPH oxidase than peripheral tissues (20). It is, therefore, conceivable that sufficient oxidative stress was present in the cerebral arteries to attenuate the NO component of the ATP dilation but not affect EDH, which is resistant to oxidative stress (8).

In summary, we have developed a new model of chronic OSA in the rat. The model incorporates apneas in freely ranging rats. The frequency of the apneas and/or the duration of each apnea can be easily manipulated to alter the severity of the OSA. We show that a frequency of apneas in rats (30/h), which is considered as moderate in humans, produced no overt systemic changes, such as hypertension over 4 wk. The lack of systemic effect in the model indicates that young healthy rats (and perhaps humans) can tolerate mild or moderate OSA (without comorbidities) for extended periods of time without overt systemic pathological changes. However, we did note that 4 wk of apnea did attenuate ATP-mediated dilations in MCA by reducing NO bioavailability. This sensitivity of the cerebral circulation to apnea likely demonstrates vulnerabilities of the cerebral circulation to OSA that may increase the probability of a cerebrovascular accident.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES


