age 24-h blood pressure level is significantly associated with cardiovascular damage, whereas the office blood pressure level is not (*2*). It has also been reported that an absence of nocturnal blood pressure fall (non-dipper phenomenon) is associated with cerebrovascular damage (*4*), whereas blood pressure lability, which is defined as increased short-term blood pressure variability and is a feature of hypertension in the elderly (*5*, *6*), has been reported to be associated with hypertensive target organ damage (*7*, *8*). However, the causal relationship between blood pressure lability and hypertensive organ damage remains unknown. To clarify this point, the direct effects of blood pressure lability on the vascular wall independent of the average blood pressure level should be investigated.

For this purpose, we selected sinoaortic-denervated rats as an animal model of blood pressure lability. The arterial baroreflex plays a pivotal role in the neural regulation of blood pressure. The afferent fibers of this negative feedback reflex arise from the carotid sinuses and aortic arch. Denervation of the afferent fibers of the baroreflex (sinoaortic denervation; SAD) was originally reported to induce neurogenic hypertension in rats (*9*). However, several studies using long-term continuous blood pressure measurement in the conscious state have shown that SAD does not affect the average blood pressure level and induces a marked increase in blood pressure variability (*10*, *11*).

The aim of this study was thus to elucidate the effects of blood pressure lability on vascular function and remodeling in SAD rats. In the first experiment, we examined the isometric tension of isolated aortic rings to investigate endothelial vasomotor function. In the second experiment, the degree of neointimal formation after balloon injury of the carotid artery was analyzed.

Methods

SAD Procedure

Ten-week-old male Wistar rats (Japan Clea, Tokyo, Japan) were used in this study. They were kept individually in stainless steel cages in a room where lighting was controlled (12 h on, 12 h off) and room temperature was maintained at around 22°C. They were given a standard diet and water *ad libitum*. The experimental protocols were approved by the Animal Research Committee of the University of Tokyo. SAD was performed according to the method of Krieger with slight modification (*9*). Briefly, rats were anesthetized with a single intraperitoneal injection of pentobarbital (50 mg/kg). A midline neck incision was made and the sternocleidomastoid muscle was retracted laterally. The cervical sympathetic trunks, the superior laryngeal nerves and the aortic depressor nerves were bilaterally isolated and resected. The carotid sinuses were stripped of all connective tissue and treated with 10% phenol. After this procedure was completed, the incision was sutured. Sham-operated rats underwent the same procedure except that afferent fibers of the baroreflex were not denervated.

Continuous Mean Arterial Pressure (MAP) Recording

The femoral artery and vein were catheterized with polyethylene tubes (PE-50 and PE-20, respectively; Becton Dickinson, Parsippany, USA). To confirm denervation, baroreflex sensitivity was evaluated after intravenous bolus injection of phenylephrine hydrochloride (6 mg/kg). MAP and heart rate were recorded continuously *via* the arterial catheter in the conscious state over a 3-h period (2 to 5 PM). Continuous recording was performed from 1 to 3 days and again at 4 weeks after SAD operation. Data were sampled every 20 s with an analog-to-digital converter and stored on a Macintosh computer. The average value and standard deviation of MAP were calculated. The standard deviation of MAP was used as an index of blood pressure lability.

Vascular Reactivity of Aortic Rings

Four weeks after SAD or sham operation, the vasoreactivity of isolated aortic rings was evaluated as described previously (*12*, *13*) with slight modification (Fig. 1A). The rats were killed with a lethal dose of anesthetic, and the thoracic aorta was removed. The aorta was dissected free of adherent fat and connective tissue and cut into rings (3 mm in length). The aortic ring was placed horizontally between L-shaped stainless wires in an organ bath chamber filled with oxygenated (95% O_2, 5% CO_2) balanced salt solution (37°C, pH 7.4) of the following composition: NaCl 112 mmol/l, KCl 4.7 mmol/l, $CaCl_2$ 0.9 mmol/l, $MgCl_2$ 1.2 mmol/l, $NaHCO_3$ 25 mmol/l, KH_2PO_4 1.2 mmol/l, glucose 11 mmol/l, and EDTA 0.026 mmol/l. The aortic ring was connected to a force transducer for isometric tension recording. After a 60-min equilibration period, the ring was gradually stretched to an optimal resting tension of 2 g. Then the ring was contracted by addition of KCl (60 mmol/l) and washed with fresh balanced salt solution. The aortic ring was allowed to equilibrate for 30 min before the experiment.

To test the vasorelaxing reactivity, the aortic ring was precontracted with norepinephrine (100 nmol/l) and then relaxed by cumulative addition of an endothelium-dependent vasodilator, acetylcholine (1 nmol/l to 10 μmol/l), or an endothelium-independent vasodilator, sodium nitroprusside (1 nmol/l to 10 μmol/l). Relaxation was expressed as a percentage of the tension induced by norepinephrine.

NO Production by Aortic Rings

Four weeks after SAD or sham operation, the isolated aortic ring was opened longitudinally and incubated in 1 ml balanced salt solution containing acetylcholine (1 μmol/l) and L-arginine (100 μmol/l) at 37°C. After 20 or 60 min of incubation, a sample of the solution was collected to analyze ni-

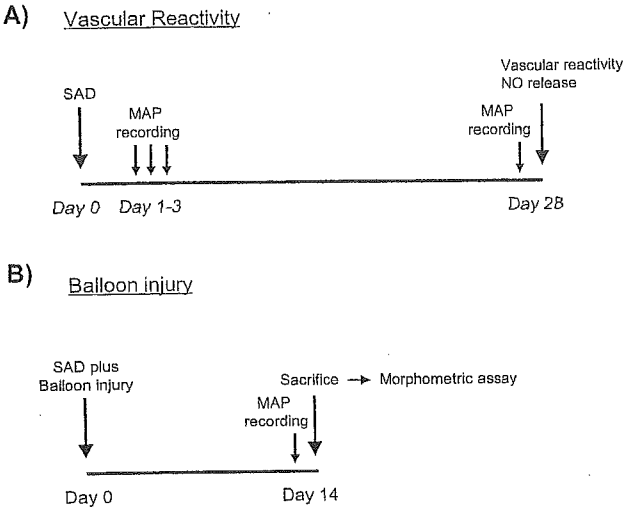

Fig. 1. *Experimental protocols for the vascular reactivity (A) and balloon injury (B). SAD, sinoaortic denervation; MAP, mean arterial pressure.*

trite/nitrate concentration as a measure of NO release. After incubation, the wet weight of the aortic ring was measured. Nitrite/nitrate concentration in the solution was determined using an automatic analyzer that employs automated flow injection analysis (TCI-NOX5000S; Tokyo Kasei Kogyo, Tokyo, Japan) as described previously (*14*). Briefly, the sample (0.1 ml) was diluted with 0.4 ml distilled water, and 0.3 ml 0.3 eq/l NaOH was added. After incubation for 5 min at room temperature, 0.3 ml 5% (w/v) $ZnSO_4$ was added, and the sample was incubated for an additional 5 min. The mixture was centrifuged at 2,800×*g* for 10 min and the supernatant was applied to the analyzer. The nitrite reacted with Griess reagent to form a purple azo compound. The absorbance at 540 nm was measured. The nitrate concentration was determined by means of reduction to nitrite through a copperized cadmium reduction column.

Balloon Injury in the Carotid Artery

In a separate experiment, balloon injury of the carotid artery and SAD (or sham operation) were performed simultaneously in order to investigate the effects of blood pressure lability on neointimal formation (Fig. 1B). Balloon injury was performed as described previously (*15*). Briefly, a 2 French Fogarty arterial embolectomy balloon catheter (Baxter, Irvine, USA) was inserted into the left common carotid artery through the left external carotid artery and advanced to the aortic arch. The balloon was inflated with saline and gradually withdrawn to the carotid bifurcation. This procedure was repeated three times, and then the balloon catheter was removed and the left external carotid artery was ligated.

The rats were killed with a lethal dose of anesthetic 14 days after balloon injury. The carotid artery was perfused and fixed with 4% paraformaldehyde at 100 mmHg and then removed for histological examination.

Morphometric Assay

Morphometric assay was performed as described previously (*16*). The middle third of the fixed carotid artery was embedded in paraffin, and multiple 5-μm cross sections were stained with hematoxylin and eosin or elastica van Gieson. After the section was photographed, the image was scanned and analyzed using NIH Image software. Then, the area of the neointima and of the media and the neointimal-to-medial area ratio were calculated. Three portions of each sample were analyzed, and the mean values were subjected to statistical analysis.

In Vivo Cell Proliferation Assay

Immunohistochemical staining of sections was carried out by the streptavidin-biotin-peroxidase method as described previously (*17*). We used anti-proliferating cell nuclear antigen (PCNA) antibody (PC10, 10 μg/ml; Boehringer Mannheim Biochemica, Mannheim, Germany) and normal mouse IgG (10 μg/ml) as the primary antibody. Specifically bound antibody was visualized by immersing the section in a substrate solution of 3,3-diaminobenzine (Vector Laboratories, Burlingame, USA). The number of positively stained nuclei within the neointima was counted, and the ratio of the number of positive cells to the total number of cells, expressed as a percentage, was calculated as an index of proliferation. Three vision fields of each sample were analyzed, and the mean values were subjected to statistical analysis.

Data Analysis

All values were expressed as the mean±SEM. In the vascular reactivity experiment, the concentration of substance (expressed as −log mol/l) evoking 50% relaxation (pD_2) and the maximum relaxation response (expressed as a percentage of precontraction response) were calculated. Unpaired Student's *t*-test was used for statistical analysis of differences between the two groups. Values of $p<0.05$ were considered to indicate statistical significance.

Results

Baroreflex sensitivity was assessed 3 days after SAD in terms of the reflex reduction in heart rate occurring in response to phenylephrine-induced increase in MAP level. When expressed as the ratio of heart rate decrease to MAP increase, the reflex response in SAD rats ($n=10$) was significantly diminished compared with that in sham-operated rats ($n=6$) (-1.09 ± 0.23 *vs.* -4.43 ± 0.96 bpm/mmHg, respectively, $p<0.01$).

The typical heart rate and MAP recordings of a SAD rat and a sham-operated rat on day 3 are shown in Fig. 2. A

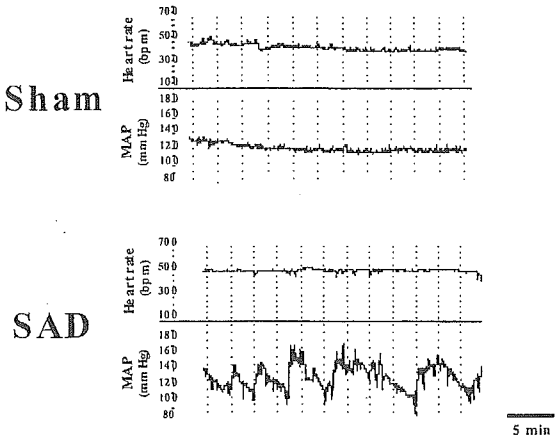

Fig. 2. *Representative heart rate and mean arterial pressure (MAP) recordings of a sinoaortic-denervated rat (SAD) and a sham-operated rat (Sham).*

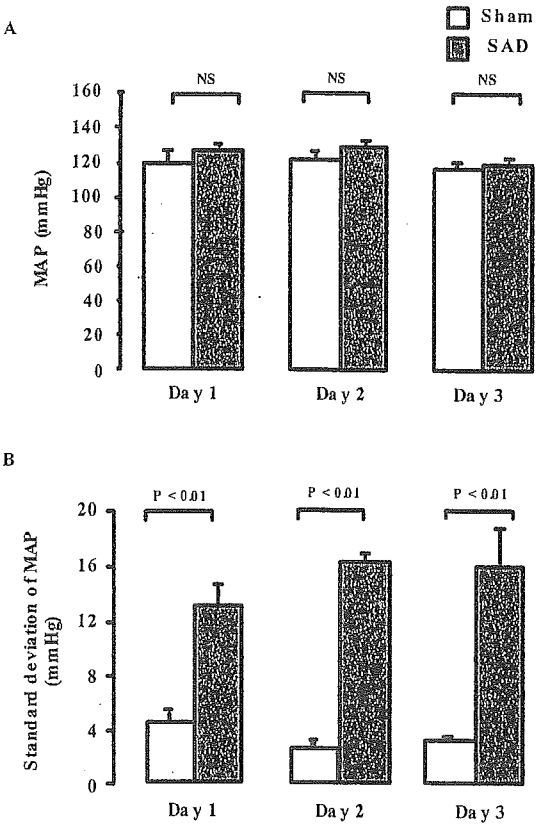

Fig. 3. *Average level of mean arterial pressure (MAP) (A) and standard deviation of MAP (B) from day 1 to day 3 after sinoaortic-denervation (SAD) or sham operation (Sham) in rats. Each bar represents the mean±SEM (n=5 for each group).*

marked increase in short-term MAP variability with an equivalent average level of MAP was observed in the SAD rat. A summary of MAP data on days 1–3 after operation is shown in Fig. 3. The average MAP level was not significantly different between SAD rats and sham-operated rats from 1 to 3 days after SAD (or sham operation) (Fig. 3A). However, the standard deviation of MAP, an index of blood pressure lability, was significantly greater in SAD rats at these time points (Fig. 3B). Table 1 shows the effects of SAD for 4 weeks on body weight, heart rate, and MAP. The body weight, heart rate, and average MAP value in sham-operated rats were identical to those in SAD rats. In contrast, the standard deviation of MAP in SAD rats remained significantly greater than that in sham-operated rats. Similar results on average MAP and standard deviation of MAP were obtained at 2 weeks after the operation (data not shown).

Four weeks after SAD or sham operation, the vasoreactivity of isolated aortic rings was evaluated. Maximum contraction in response to norepinephrine (100 nmol/l) was similar in the two groups (Table 2). Endothelium-dependent relaxation in response to acetylcholine was impaired in the SAD group (Fig. 4A). Table 2 shows that both maximum relaxation and sensitivity (pD_2) in response to acetylcholine were

Table 1. Effects of Sinoaortic Denervation for 4 Weeks on Body Weight, Heart Rate and Mean Arterial Pressure in Conscious Rats

	Sham	SAD
Body wegiht (g)	440±12	400±12
Heart rate (bpm)	447±28	486±21
Mean arterial pressure (mmHg)		
Average	121±5.3	124±4.0
Standard deviation	4.7±0.9	13.3±0.9*

Values are expressed as mean±SEM of 5–7 animals. Sham, sham-operated rats; SAD, sinoaortic-denervated rats. * $p<0.01$ *vs.* sham-operated rats.

Table 2. Maximum Response and Sensitivity (pD_2) to Acetylcholine and Sodium Nitroprusside in Isolated Aortic Rings of SAD and Sham

	Sham	SAD
Maximum contraction to norepinephrine (g)	0.84±0.15	0.87±0.05
Acetylcholine		
Maximum relaxation (% of precontraction)	90.6±3.0	66.6±2.2*
pD_2 (−log mol/l)	7.21±0.26	6.44±0.16*
Sodium nitroprusside		
Maximum relaxation (% of precontraction)	98.3±1.3	95.8±1.6
pD_2 (−log mol/l)	6.94±0.12	6.69±0.10

Values are expressed as mean±SEM of 5–7 animals. Sham, sham-operated rats; SAD, sinoaortic-denervated rats. pD_2, concentration evoking 50% relaxation. * $p<0.05$ *vs.* sham-operated rats.

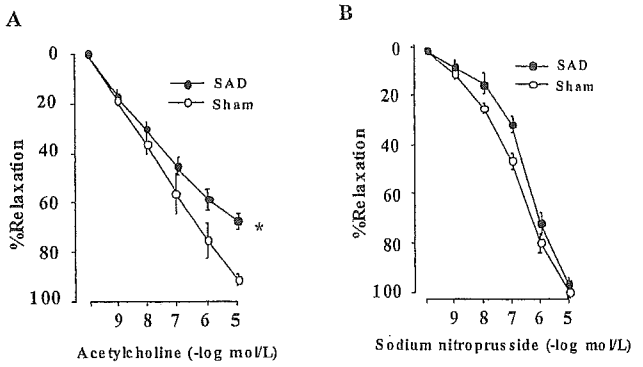

Fig. 4. *Endothelium-dependent relaxation response to acetylcholine (A) and endothelium-independent relaxation response to sodium nitroprusside (B) in isolated aortic rings of sinoaortic-denervated rats (SAD) and sham-operated rats (Sham). Data are expressed as the percent relaxation of contraction evoked by norepinephrine (100 nmol/l) and shown as the mean±SEM* (n=*5–7 for each group).* * $p<0.05$ vs. *sham-operated rats for maximum relaxation.*

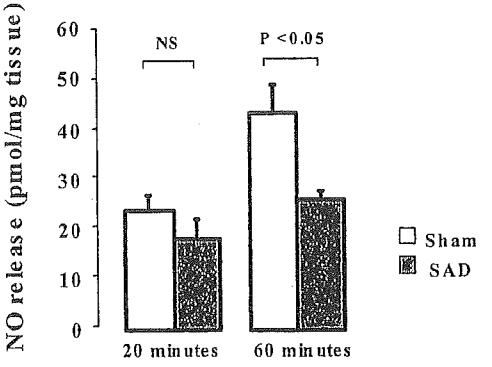

Fig. 5. *Acetylcholine (1 μmol/l)-induced NO production by isolated aortic rings derived from sinoaortic-denervated rats (SAD) and sham-operated rats (Sham). Each bar represents the mean±SEM* (n=*5–7 for each group).*

significantly reduced in the SAD group compared with the values in the sham-operated group (Fig. 4A, Table 2). On the other hand, endothelium-independent relaxation in response to sodium nitroprusside was not significantly different between the SAD group and sham-operated group (Fig. 4B). As shown in Table 2, the maximum relaxation response to sodium nitroprusside was identical in the two groups. Sensitivity to sodium nitroprusside tended to be slightly impaired in the SAD group; however, this difference was not statistically significant (Table 2).

To analyze endothelial NO release from isolated endothelium-preserved aortic rings, the nitrite/nitrate concentration was measured in a balanced salt solution containing acetylcholine (1 μmol/l) 4 weeks after SAD or sham operation. As shown in Fig. 5, acetylcholine-induced NO production by aortic rings was significantly reduced in the SAD group at 60 min after stimulation compared with that in the sham-operated group.

To investigate the effects of SAD on neointimal formation, SAD (or sham operation) and balloon injury were performed simultaneously, and then the injured carotid arteries were isolated 2 weeks after the procedures. Representative photomicrographs are shown in Fig. 6A and B. The neointimal area and neointimal-to-medial area ratio were significantly increased in the SAD group compared with those in the sham-operated group (Fig. 7A, C), while no significant difference was observed in medial area between the two groups (Fig. 7B). To evaluate cell-proliferating activity *in vivo*, immunohistochemical staining using anti-PCNA antibody was performed (Fig. 6C, D). The percentage of PCNA-positive cells in the neointima was significantly increased in the SAD group compared with that in the sham-operated group (SAD group, 11.8±1.0%; sham-operated group, 8.2±1.0%; $n=6$, $p<0.05$).

Discussion

SAD was previously reported to cause neurogenic hypertension (*9*). However, in that study, blood pressure was measured indirectly in restrained rats, and hypertension may have been attributable to the acute response to restraint rather than the effect of SAD. Several recent studies have reported an absence of sustained hypertension in SAD animals using direct measurement over a long period (*10*, *11*). These reports concluded that SAD induces a marked increase in blood pressure lability without affecting the average blood pressure level. Consistent with these findings, our data demonstrated that blood pressure lability (standard deviation of MAP) was significantly increased without elevation of average MAP in SAD rats from the initial phase (days 1–3) to the chronic phase (4 weeks after SAD). Therefore, we considered that SAD rats are appropriate as an animal model of blood pressure lability to evaluate the effects of blood pressure lability on the vascular wall *in vivo*.

Recent studies have shown that endothelium-derived NO is not only a potent vasorelaxing factor, but that it also exerts an antiatherogenic action by inhibiting the migration and proliferation of vascular smooth muscle cells (*18*, *19*), monocyte adhesion to the endothelium (*20*), and platelet aggregation (*21*, *22*). In addition, endothelial dysfunction has been considered to be an important process in the early stage of atherosclerosis (*23*). Therefore, it is of critical importance to investigate whether endothelial dysfunction is present in this animal model of blood pressure lability. In the present study, the endothelium-dependent relaxing response of isolated aortic rings from SAD rats was significantly decreased compared with that in sham-operated control rats, whereas the endothelium-independent relaxation responses by sodium nitroprusside were comparable between the two groups. Although similar results were reported recently (*24*), the mechanisms underlying impaired endothelium-dependent vasomotor function in SAD rats have not been elucidated. To

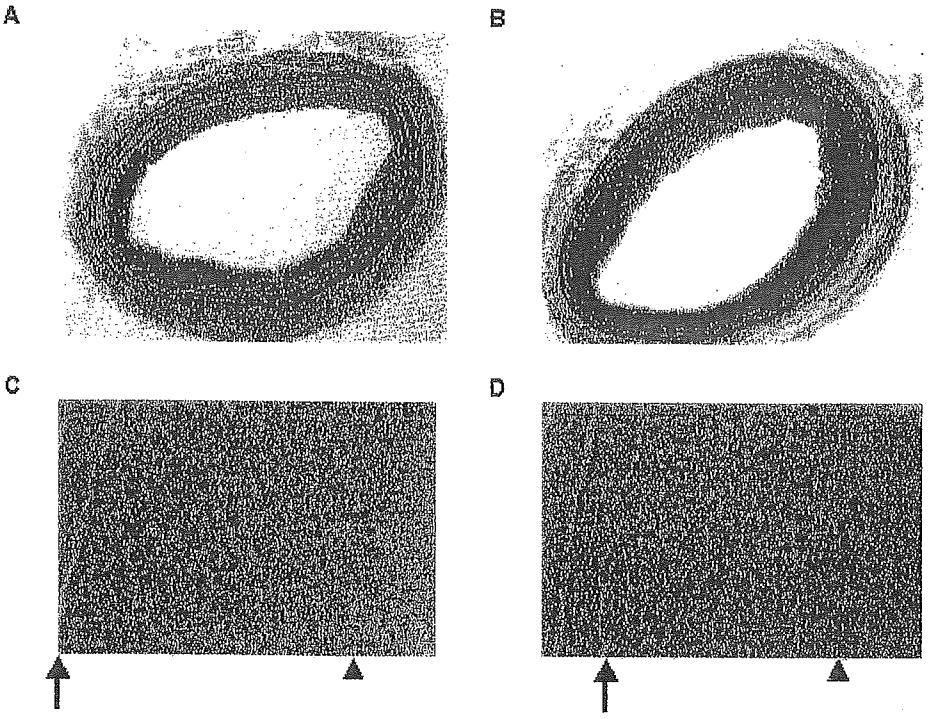

Fig. 6. *Representative histological sections of common carotid arteries 14 days after balloon injury from a sham-operated rat (A, C) and a sinoaortic-denervated rat (SAD) (B, D). Elastica van Gieson staining (A, B) and immunohistochemical staining using anti-proliferating cell nuclear antigen (C, D). The arrow indicates the internal elastic lamina and the arrowhead indicates the luminal margin. Magnification, ×100 (A, B) and ×400 (C, D).*

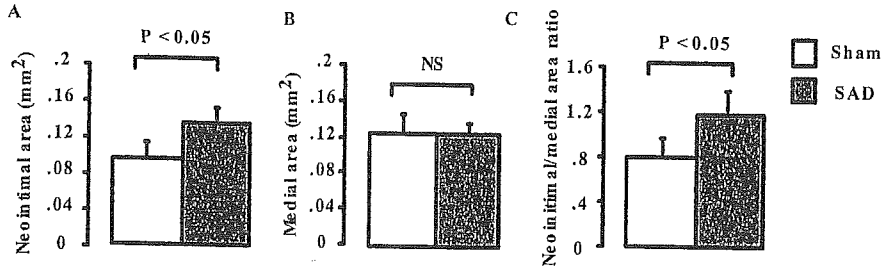

Fig. 7. *A and B show cross-sectional areas of the neointima and media, respectively, of carotid arteries 14 days after balloon injury from sinoaortic-denervated rats (SAD) and sham-operated rats (Sham). C shows the neointimal-to-medial area ratio of carotid arteries from the same two groups. Each bar represents the mean±SEM (*n=*6 for each group).*

clarify this point, we also evaluated NO production from isolated aortas. Acetylcholine-induced NO release from endothelium-preserved aortic rings was also significantly decreased in SAD rats. Since NO release is not stimulated by acetylcholine in endothelium-deprived aortic rings (*25*), endothelial NO production is considered to be reduced in SAD rats. Other possible mechanisms of impairment of endothelial function in SAD rats should be examined. As is the case with endothelial dysfunction in animal models of hypertension (*26, 27*), several mechanisms can be hypothesized, such as augmented endothelium-derived constricting factor, increased NO inactivation (augmented superoxide production), and desensitization of vascular smooth muscle to endothelium-derived vasorelaxing factor. In fact, mechanical stretch stimulates superoxide production in cultured vascular endothelial cells (*28*). To address these issues, further investigation is needed, including the measurement and blockade of superoxide and endothelium-derived constricting factor in SAD rats. However, taking these results together, we can conclude that SAD itself causes endothelial dysfunction in rats, at least in part by inhibiting endothelial NO production.

In the second set of experiments, we examined whether SAD affects the vascular structural change induced by balloon injury. Neointimal formation after balloon injury was significantly enhanced in SAD rats compared with that in sham-operated rats. It has been reported that atherosclerotic

plaque in the aorta induced by both high cholesterol diet and endothelial denudation is significantly enhanced in SAD rats (*29*). However, the underlying mechanisms could not be elucidated in this previous study. Neointimal formation is known to be attributable to medial smooth muscle cell migration and proliferation and accumulation of extracellular matrix (*15*, *30*). PCNA-positive cells in the neointima, as a marker of cell proliferation, were significantly increased in the SAD group compared with those in the sham-operated group. This finding indicates that the proliferation of smooth muscle cells stimulated by balloon injury was increased in the SAD group. Since NO has an inhibitory action on smooth muscle cell migration and proliferation (*18*, *19*), decreased NO production by regenerated endothelial cells after vascular injury in SAD rats may be involved in the mechanism. Unfortunately, the time courses were different between the first and the second part of the experiments, and we have no direct evidence to clarify this point in this study. Alternatively, there may be another mechanism. Blood pressure lability might directly influence smooth muscle cells by modulating the growth or production of growth factors such as transforming growth factor-β and platelet-derived growth factor. These growth factors play important roles in balloon injury-induced neointimal formation (*31–34*) and are induced by mechanical stretch in cultured vascular smooth muscle cells (*35*, *36*). Therefore, it would be interesting to study the production of these growth factors by vascular smooth muscle cells in SAD rats in the future.

It has been reported that SAD does not affect the resting plasma concentrations of neurohumoral factors such as norepinephrine (*37*), epinephrine (*37*), renin activity (*37*, *38*), and arginine vasopressin (*38*). However, since local secretion of these factors or the involvement of other neurohumoral factors is not known, a possibility may still remain that a change in neurohumoral factors after SAD affects the disturbance of endothelial function and enhanced neointimal formation.

Recent studies have focused on the clinical significance of blood pressure variability. It has been shown that blood pressure variability has several patterns according to the cycle length. Among these patterns, the circadian rhythm, which has a cycle length of approximately 24 h, has been shown to have clinical value (*4*, *39*, *40*). Similarly, previous studies (*7*, *8*, *41*) from our and other groups have sought to clarify the significance of short-term blood pressure variability, which is modulated by the arterial baroreflex. These studies have shown that increased short-term blood pressure variability (blood pressure lability) is associated with hypertensive target organ damage (*7*, *8*), cognitive function (*41*), and cardiovascular events (our unpublished observations). However, these clinical data showed only an association and could not establish a cause-and-effect relation. Recently, it has been reported that SAD rats show aortic hypertrophy (*24*, *42*), vasomotor dysfunction (*24*), and cardiac hypertrophy (*43*, *44*) without an increase in MAP, suggesting the possibility that an increase in short-term blood pressure variability may be the cause of cardiovascular damage rather than its consequence. Our present results provide additional experimental evidence in support of this hypothesis. In this context, it might be presumed that short-term blood pressure variability should be taken into consideration in the treatment of hypertensive patients, and particularly in the treatment of elderly hypertensive patients, in order to prevent target organ damage.

In summary, the present study demonstrated that SAD reduced endothelium-dependent vasorelaxation and enhanced neointimal formation after balloon injury. These results suggest that increased blood pressure lability, independently of average blood pressure level, may contribute to atherogenesis.

Acknowledgements

We thank Ms. Hitomi Yamaguchi for her excellent technical assistance.

References

1. Kannel WB: Blood pressure as a cardiovascular risk factor: prevention and treatment. *JAMA* 1996; **275**: 1571–1576.
2. Shimada K, Kawamoto A, Matsubayashi K, Ozawa T: Silent cerebrovascular disease in the elderly: correlation with ambulatory pressure. *Hypertension* 1990; **16**: 692–699.
3. Appel LJ, Stason WB: Ambulatory blood pressure monitoring and blood pressure self-measurement in the diagnosis and management of hypertension. *Ann Intern Med* 1993; **118**: 867–882.
4. Shimada K, Kawamoto A, Matsubayashi K, Nishinaga M, Kimura S, Ozawa T: Diurnal blood pressure variations and silent cerebrovascular damage in elderly patients with hypertension. *J Hypertens* 1992; **10**: 875–878.
5. Floras JS, Hassan MO, Jones JV, Osikowska BA, Sever PS, Sleight P: Factors influencing blood pressure and heart rate variability in hypertensive humans. *Hypertension* 1988; **11**: 273–281.
6. Convanico V, De Caprio L, Vigorito C, *et al*: Differences in blood pressure profile between young and elderly hypertensive patients. *J Hum Hypertens* 1990; **4**: 405–409.
7. Parati G, Pomidossi G, Albini F, Malaspina D, Mancia G: Relationship of 24-hour blood pressure mean and variability to severity of target-organ damage in hypertension. *J Hypertens* 1987; **5**: 93–98.
8. Frattola A, Parati G, Cuspidi C, Albini F, Mancia G: Prognostic value of 24-hour blood pressure variability. *J Hypertens* 1993; **11**: 1133–1137.
9. Krieger EM: Neurogenic hypertension in the rat. *Circ Res* 1964; **15**: 511–521.
10. Saito M, Terui N, Numao Y, Kumada M: Absence of sustained hypertension in sinoaortic-denervated rabbits. *Am J Physiol* 1986; **251**: H742–H747.
11. Osborn JW, England SK: Normalization of arterial pressure after barodenervation: role of pressure natriuresis. *Am J Physiol* 1990; **259**: R1172–R1180.

12. Kung CF, Moreau P, Takase H, Luscher TF: L-NAME hypertension alters endothelial and smooth muscle function in rat aorta: prevention by trandolapril and verapamil. *Hypertension* 1995; **26**: 744–751.
13. Moreau P, Takase H, Kung CF, Shaw S, Luscher TF: Blood pressure and vascular effects of endothelin blockade in chronic nitric oxide-deficient hypertension. *Hypertension* 1997; **29**: 763–769.
14. Akishita M, Kozaki K, Saito T, *et al*: Effects of estrogen on atherosclerosis formation and serum nitrite/nitrate concentrations in cholesterol-fed ovariectomized rabbits. *J Atheroscler Thromb* 1996; **3**: 114–119.
15. Clowes AW, Reidy MA, Clowes MM: Kinetics of cellular proliferation after arterial injury. *Lab Invest* 1983; **49**: 327–333.
16. Minamino T, Kurihara H, Takahashi M, *et al*: Endothelin-converting enzyme expression in the rat vascular injury model and human coronary atherosclerosis. *Circulation* 1997; **95**: 221–230.
17. Akishita M, Ouchi Y, Miyoshi H, *et al*: Estrogen inhibits cuff-induced intimal thickening of rat femoral artery: effects on migration and proliferation of vascular smooth muscle cells. *Atherosclerosis* 1997; **130**: 1–10.
18. Dubey RK, Jackson EK, Lücher TF: Nitric oxide inhibits angiotensin II-induced migration of rat aortic smooth muscle cell: role of cyclic-nucleotides and angiotensin 1 receptors. *J Clin Invest* 1995; **96**: 141–149.
19. Garg UC, Hassid A: Nitric oxide-generating vasodilators and 8-bromocyclic guanosine monophosphate inhibit mitogenesis and proliferation of cultured rat vascular smooth muscle cells. *J Clin Invest* 1989; **83**: 1774–1777.
20. Bath PMW, Hassall DG, Gladwin A-M, Palmer RMJ, Martin JF: Nitric oxide and prostacyclin: divergence of inhibitory effects on monocyte chemotaxis and adhesion to endothelium *in vitro*. *Arterioscler Thromb* 1991; **11**: 254–260.
21. Radomski MW, Palmer RMJ, Moncada S: Comparative pharmacology of endothelium-derived relaxing factor, nitric oxide, and prostacyclin in platelets. *Br J Pharmacol* 1987; **92**: 181–187.
22. Stamler JS, Mendelsohn ME, Amarante P, *et al*: *N*-Acetylcysteine potentiates platelet inhibition by endothelium-derived relaxing factor. *Circ Res* 1989; **65**: 789–795.
23. Ross R: The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature* 1993; **362**: 801–809.
24. Miao CY, Tao X, Gong K, Zhang SH, Chu ZX, Su DF: Arterial remodeling in chronic sinoaortic-denervated rats. *J Cardiovasc Pharmacol* 2001; **37**: 6–15.
25. van der Zee R, Murohara T, Luo Z, *et al*: Vascular endothelial growth factor/vascular permeability factor augments nitric oxide release from quiescent rabbit and human vascular endothelium. *Circulation* 1997; **95**: 1030–1037.
26. Wever RMF, Lücher TF, Cosentino F, Rebelink TJ: Atherosclerosis and two factors of endothelial nitric oxide synthase. *Circulation* 1998; **97**: 108–112.
27. Cooke JP, Dzau VJ: Derangements of the nitric oxide synthase pathway, L-arginine, and cardiovascular diseases. *Circulation* 1997; **96**: 379–382.
28. Hishikawa K, Lücher TF: Pulsatile stretch stimulates superoxide production in human aortic endothelial cells. *Circulation* 1997; **96**: 3610–3616.
29. Sasaki S, Yoneda Y, Fujita H, *et al*: Association of blood pressure variability with induction of atherosclerosis in cholesterol-fed rats. *Am J Hypertens* 1994; **7**: 453–459.
30. Clowes AW, Reidy MA, Clowes MM: Mechanisms of stenosis after arterial injury. *Lab Invest* 1983; **49**: 208–215.
31. Majesky MW, Lindner V, Twardzik DR, Schwartz SM, Reidy MA: Production of transforming growth factor β1 during repair of arterial injury. *J Clin Invest* 1991; **88**: 904–910.
32. Wolf YG, Rasmussen LM, Ruoslahti E: Antibodies against transforming growth factor β1 suppress intimal hyperplasia in a rat model. *J Clin Invest* 1994; **93**: 1172–1178.
33. Majesky MW, Reidy MA, Bowen-Pope DF, Hart CE, Wilcox JN, Schwartz SM: PDGF ligand and receptor gene expression during repair of arterial injury. *J Cell Biol* 1990; **111**: 2149–2158.
34. Ferns GAA, Raines EW, Sprugel KH, Motani AS, Reidy MA, Ross R: Inhibition of neointimal smooth muscle accumulation after angioplasty by an antibody to PDGF. *Science* 1991; **253**: 1129–1132.
35. Wilson E, Mai Q, Sudhir K, Weiss RH, Ives HE: Mechanical strain induces growth of vascular smooth muscle cells *via* autocrine action of PDGF. *J Cell Biol* 1993; **123**: 741–747.
36. Li Q, Muragaki Y, Hatamura I, Ueno H, Ooshima A: Stretch-induced collagen synthesis in cultured smooth muscle cells from rabbit aortic media and a possible involvement of angiotensin II and transforming growth factor-beta. *J Vasc Res* 1998; **35**: 93–103.
37. Buchholz RA, Hubbard JW, Keeon TK, Nathan MA: Cardiovascular and neuroendocrine responses to behavioral stress after central or peripheral barodenervation in rats. *Brain Res* 1986; **365**: 360–364.
38. Quail AW, Woods RL, Korner PI: Cardiac and arterial baroreceptor influences in release of vasopressin and renin during hemorrhage. *Am J Physiol* 1987; **252**: H1120–H1126.
39. Kario K, Matsuo T, Kobayashi H, Imiya M, Matsuo M, Shimada K: Nocturnal fall of blood pressure and silent cerebrovascular damage in elderly hypertensive patients: advanced silent cerebrovascular damage in extreme dippers. *Hypertension* 1996; **27**: 130–135.
40. Verdecchia P, Shillaci G, Guerrieri M, *et al*: Circadian blood pressure changes and left ventricular hypertrophy in essential hypertension. *Circulation* 1990; **81**: 528–536.
41. Kanemaru A, Kanemaru K, Kuwajima I: The effects of short-term blood pressure variability and nighttime blood pressure levels on cognitive function. *Hypertens Res* 2001; **24**: 19–24.
42. Lacolley P, Bezie Y, Girerd X, *et al*: Aortic distensibility and structural changes in sinoaortic-denervated rats. *Hypertension* 1995; **26**: 337–340.
43. Miao CY, Su DF: The importance of blood pressure variability in rat aortic and left ventricular hypertrophy produced by sinoaortic denervation. *J Hypertens* 2002; **20**: 1865–1872.
44. Van Vliet BN, Hu L, Scott T, Chafe L, Montani JP: Cardiac hypertrophy and telemetered blood pressure 6 wk after baroreceptor denervation in normotensive rats. *Am J Physiol* 1996; **271**: R1759–R1769.

Geriatrics and Gerontology International 2003; **3:** *145–149*

ORIGINAL ARTICLE

Influence of sex and estrogen on vitamin D-induced arterial calcification in rats

Tetsuro Nakamura,[1] Masahiro Akishita,[2] Koichi Kozaki,[3] Kenji Toba,[2] Hajime Orimo[4] and Yasuyoshi Ouchi[3]

[1]Research Institute of Aging Science, [2]Department of Geriatric Medicine, Kyorin University School of Medicine, [3]Department of Geriatric Medicine, Graduate School of Medicine, University of Tokyo and [4]Tokyo Metropolitan Geriatric Hospital, Tokyo, Japan

Background: It is known that the process of arteriosclerosis is affected by sex and estrogen. The present study was thus undertaken to examine the effects of these factors on arterial calcification, a form of arteriosclerosis, using a rat model of vitamin D toxicity.

Methods and results: Vehicle or 5 μg/kg per day 1α(OH)D_3 was given to male and female 30-week-old Fisher rats for 2 weeks. Arterial calcification, evaluated by calcium content in the aorta, was 70% more marked in male rats compared to that in female rats, whereas calcium content in the aorta was similar in vehicle-treated male and female rats. Next, the effects of ovariectomy and estrogen replacement (estradiol dipropionate 20 μg/kg per week) were examined in female rats given 5 μg/kg per day 1α(OH)D_3 for 2 weeks. Calcium content in the aorta was significantly higher in ovariectomized rats than in sham-operated rats and in ovariectomized and estrogen-replaced rats. No difference between the groups was seen when vehicle was given to the animals.

Conclusions: These results suggest that sex and estrogen can modify the process of arterial calcification. The mechanisms remain to be determined, although the effects were independent of serum calcium level.

Keywords: artery, estradiol, gender, histo(patho)logy, hormones, smooth muscle.

Introduction

Arterial calcification is an important manifestation of arteriosclerosis that reflects advanced atheromatous disease[1,2] and predicts coronary events.[3] Arterial calcification independent of atherosclerosis can be induced in experimental animals by vitamin D toxicity although calcification is confined to the media and resembles the pattern seen in Monckeberg's syndrome.[4,5] This type of arterial calcification is frequently seen in dialysis patients with chronic renal failure[6,7] and in the legs of diabetic patients,[8,9] and is associated with cardiovascular disease.[7,9] Thus, vitamin D toxicity-induced arterial calcification is considered not only an animal model but also a useful tool to explore the pathogenesis of arterial calcification in humans.

A number of epidemiological studies have shown that the prevalence of cardiovascular disease is lower in premenopausal women than in men of the same age but increases after menopause.[10,11] According to observational studies, estrogen replacement protects postmenopausal women from coronary heart disease,[10,11] although the cardioprotective effects of estrogen plus progestin replacement have not been found in randomized controlled studies.[12,13] The sex difference and benefit of estrogen in coronary heart disease are largely attributable to the impact on atherosclerosis through various mechanisms such as lipid metabolism and vascular function.[10,11,14] Regarding arterial calcification, a lower prevalence in women[15,16] and inhibition by estrogen replacement[17,18] are also reported in observational

Accepted for publication 29 January 2003.

Correspondence: Yasuyoshi Ouchi, MD, PhD, Department of Geriatric Medicine, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan.
Email: youchi-tky@umin.ac.jp

studies. These findings may result from the difference in atherosclerotic plaque burden, but suggest a possible role of sex and estrogen in the process of arterial calcification. In fact, it is reported that the sex difference in arterial calcification and the effect of estrogen replacement were independent of atherosclerotic plaque size.[16,18]

Thus, in the present study we tested the hypothesis that female rats would be resistant to arterial calcification compared to male rats, and that estrogen would inhibit arterial calcification in females. For this purpose we applied a toxic dose of vitamin D and induced arterial calcification in rats.

Methods

Maintenance of animals

Eight-week-old-and 30-week-old male and female Fisher rats (Nippon Bio-Supply Center, Tokyo, Japan) were used in the present study. They were kept individually in stainless steel cages in a room where lighting was controlled (12 h on, 12 h off) and room temperature was kept at 22°C. They were given a standard diet (CE-2; Japan Clea, Tokyo, Japan) and water ad libitum. The diet contained 1.2% calcium and 1.08% phosphorus by weight. All the surgical procedures were performed under continuous ether anesthesia. All of the experimental protocols were approved by the Animal Research Committee of the University of Tokyo.

Experimental groups

In the first set of experiments, 8-week-old and 30-week-old male rats were used and divided into three groups. Two groups were orally given 5 or 10 μg/kg 1α(OH)D_3 (dissolved in 0.5 mL corn oil; supplied by Teijin, Tokyo, Japan) using a feeding tube daily for 2 weeks. The other group of rats was orally given corn oil as vehicle for 2 weeks.

Sex difference in arterial calcification was examined using 30-week-old male and female rats. They were orally given vehicle or 5 μg/kg 1α(OH)D_3 daily for 2 weeks. To examine the effect of estrogen on arterial calcification, 8-week-old female rats were randomly divided into three groups.[19] Two groups of rats were ovariectomized and the other group received a sham operation. After a 1 week recovery period, each group was orally given vehicle or 5 μg/kg 1α(OH)D_3 daily for 2 weeks. At the same time, one group of ovariectomized rats received a subcutaneous injection of estradiol dipropionate (20 μg/kg; Teikoku Hormone, Tokyo, Japan) suspended in corn oil once a week (OVX + E2 group). The other group of ovariectomized rats (OVX group) and sham-operated rats (Sham group) received the same amount of corn oil as vehicle once a week.

After the experimental period, blood was collected to measure serum calcium concentration, the rats were killed by exsanguination, and the aorta was excised from the root to the iliac bifurcation.

Assay for arterial calcification and serum calcium

The aorta was dissected free from the surrounding adventitial tissue using a cotton swab, and was then lyophilized and weighed. The lyophilized aorta was burnt in an electric oven (700°C for 24 h), and the ashes were dissolved in HCl. Calcium concentration in these aliquots and in the serum was determined with an atomic absorption spectrometer (Model 180–60; Hitachi, Tokyo, Japan).[20] Calcium content in the aorta was normalized by dry weight and used as a measure of arterial calcification.

Data analysis

The values in the text, tables and figures are expressed as mean ± SEM. The data were analyzed using one-factor ANOVA. If a statistically significant effect was found, Bonferroni test was performed to isolate the difference between the groups. $P < 0.05$ was considered statistically significant.

Results

Effect of age and dosage of vitamin D

Vehicle or 1α(OH)D_3 (5 or 10 μg/kg per day) was given to 8-week-old and 30-week-old male rats for 2 weeks. Administration of 1α(OH)D_3 raised serum calcium concentration similarly in the two age groups; 9.8 ± 0.5 mg/dL for vehicle, 12.7 ± 0.4 mg/dL at 5 μg/kg per day and 13.0 ± 0.4 mg/dL at 10 μg/kg per day in 8-week-old rats ($n = 5$, $P < 0.01$); 9.5 ± 0.4 mg/dL for vehicle, 12.0 ± 0.5 mg/dL at 5 μg/kg per day and 12.1 ± 0.6 mg/dL at 10 μg/kg per day in 30-week-old rats ($n = 5$, $P < 0.01$). There was no significant difference in serum calcium concentration between 5 and 10 μg/kg per day 1α(OH)D_3 and between 8-week-old and 30-week-old rats. Calcium content in the aorta was increased by vitamin 1α(OH)D_3 administration in a dose-dependent manner (Fig. 1). Interestingly, arterial calcification was exaggerated in 30-week-old male rats compared to 8-week-old-male rats at 5 μg/kg per day 1α(OH)D_3 but not at 10 μg/kg per day and with vehicle, suggesting that age affects arterial calcification depending on the experimental conditions. In these series of experiments, histological examination (hematoxylin and eosin staining and von Kossa staining) confirmed that calcification was confined to the media as previously reported[4,5] (data not shown).

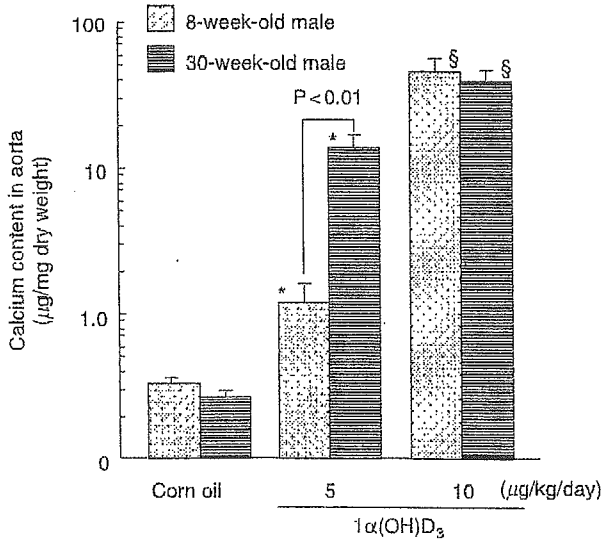

Figure 1 Influence of age and vitamin D dosage on arterial calcification in male rats. Vehicle or $1\alpha(OH)D_3$ (5 or 10 μg/kg/per ay) was given to 8-week-old and 30-week-old male rats for 2 weeks. Calcium content in the aorta was then measured and normalized by dry weight. $^*P < 0.01$ *vs* corn oil, $^{\S}P < 0.01$ *vs* corn oil and 5 μg/kg per day $1\alpha(OH)D_3$. $n = 5$ for each group.

Sex difference and effects of estrogen

Sex difference was examined in male and female 30-week-old rats. The rats were given vehicle or 5 μg/kg per day $1\alpha(OH)D_3$ for 2 weeks. Serum estradiol concentration was 38 ± 7 pmol/L for vehicle and 35 ± 6 pmol/L for $1\alpha(OH)D_3$ in male rats, and 128 ± 31 pmol/L for vehicle and 120 ± 35 pmol/L for $1\alpha(OH)D_3$ in female rats ($P < 0.05$ *vs* male). Serum calcium concentration was not different between male and female rats although $1\alpha(OH)D_3$ raised serum calcium concentration significantly in each sex; 9.8 ± 0.2 mg/dL for vehicle and 13.2 ± 0.2 mg/dL for $1\alpha(OH)D_3$ in male rats ($P < 0.01$); 10.3 ± 0.1 mg/dL for vehicle and 14.4 ± 0.3 mg/dL for $1\alpha(OH)D_3$ in female rats ($P < 0.01$). Calcium content in the aorta in vitamin D-treated rats was 70% higher in male than in female rats, whereas they were similar in vehicle-treated male and female rats (Fig. 2).

To further examine the sex-related effects on arterial calcification, ovariectomy and estrogen replacement were performed in female rats. In this set of experiments, younger 8-week-old rats were given vehicle or 5 μg/kg per day $1\alpha(OH)D_3$ for 2 weeks. Neither ovariectomy nor ovariectomy plus estrogen replacement influenced the serum calcium level (Table 1). However, arterial calcification in response to vitamin D toxicity was significantly exaggerated in OVX compared to Sham and OVX + E2 (Fig. 3). No difference between the groups was seen when vehicle corn oil was given to the animals.

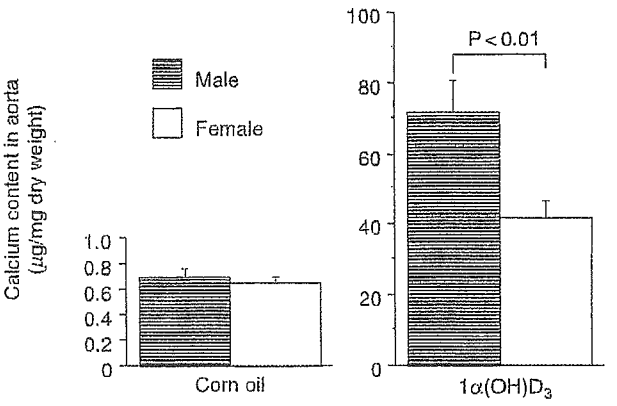

Figure 2 Sex difference in vitamin D toxicity-induced arterial calcification in rats. Vehicle or 5 μg/kg per day $1\alpha(OH)D_3$ was given to 30-week-old male and female rats for 2 weeks. Calcium content in the aorta was then measured and normalized by dry weight. $n = 8$ for corn oil and $n = 13$ for $1\alpha(OH)D_3$ treatment.

Discussion

Vitamin D toxicity-induced calcification is seen in various organs including artery, trachea, lung, kidney and intestine,[5,21] indicating that calcification is a systemic occurrence. The site of vitamin D-induced arterial calcification, however, is restricted to the medial layer.[4,5] This is different from atheromatous intimal calcification in terms of localization but clinically important as well, particularly in patients with end-stage renal disease[6,7] and in diabetic patients.[8,9] A few factors are known to affect vitamin D-induced arterial calcification: bisphosphonates[21] and osteoprotegerin[5] both inhibit arterial calcification and bone resorption. In the present study we demonstrated that sex and estrogen influenced vitamin D-induced arterial calcification in rats.

In the first set of experiments we applied different doses of vitamin $1\alpha(OH)D_3$ in two age groups of male rats. The degree of arterial calcification was dependent on the dose of vitamin D, and was higher in 30-week-old rats than in 8-week-old rats when 5 μg/kg per day $1\alpha(OH)D_3$ was administered. This is consistent with the decreased regression of arterial calcification in 30-week-old rats compared to 8-week-old rats (our preliminary results; data not shown); calcium content in the aorta was measured after 2 week administration of 10 μg/kg per day $1\alpha(OH)D_3$ and a 3 week washout period. These results suggest an effect of age or aging on arterial calcification, but are too preliminary to form a conclusion on the issue of age. Older and younger age groups should be added in the analysis.

Based on the previous experiment, sex difference was examined using 5 μg/kg per day $1\alpha(OH)D_3$ and 30-week-old male and female rats. Arterial calcification in response to vitamin D was exaggerated in male rats

Table 1 Serum calcium concentration in sham, OVX and OVX + E2 female rats

	Treatment group	Serum calcium (mg/dL)	Estradiol (pmol/L)
Sham	Corn oil	10.8 ± 0.2	129 ± 30
	$1\alpha(OH)D_3$	14.2 ± 0.3*	124 ± 33
OVX	Corn oil	10.7 ± 0.1	40 ± 6§
	$1\alpha(OH)D_3$	14.8 ± 0.3*	37 ± 7§
OVX + E2	Corn oil	10.9 ± 0.2	95 ± 15
	$1\alpha(OH)D_3$	14.8 ± 0.2*	90 ± 20

Sham, sham-operated; OVX, ovariectomized; OVX + E2, OVX + estradiol-replaced. Values are expressed as mean ± SEM. *P < 0.01 vs corn oil; §P < 0.05 *vs* Sham. $n = 8$ for corn oil and $n = 2$ for $1\alpha(OH)D_3$ treatment.

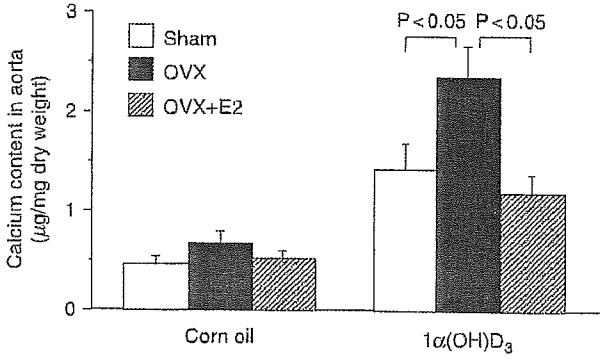

Figure 3 Effects of ovariectomy and estrogen replacement in vitamin D toxicity-induced arterial calcification in female rats. Vehicle or 5 μg/kg per day $1\alpha(OH)D_3$ was given to 8-week-old sham-operated (Sham), ovariectomized (OVX), and OVX + estradiol-replaced (OVX + E2) female rats for 2 weeks. Calcium content in the aorta was then measured and normalized by dry weight. $n = 8$ for corn oil and $n = 12$ for $1\alpha(OH)D_3$ treatment.

compared to female rats with similar serum calcium level and baseline calcium content in the aorta. Because estrogen plays an important role in sex difference and atherogenesis,[10,11,14] the effects of ovariectomy and estrogen replacement were examined. Younger 8-week-old female rats were used in the present experiment. Accordingly, the calcification response to 5 μg/kg per day vitamin D was smaller than that in 30-week-old female rats. Estrogen status does not account for this phenomenon because serum estradiol level was comparable in 8-week-old and 30-week-old female rats. Ovariectomy increased and estrogen replacement reversed arterial calcification. Conversely, ovariectomy reduced serum estradiol concentration to the level of that in male rats, and estrogen replacement raised it to the level of that in control female rats. Serum calcium level was not influenced by ovariectomy or estrogen replacement.

Our results concerning sex difference and the effect of estrogen on arterial calcification fit with the clinical observations that the prevalence and degree of coronary calcium detected by ultrafast computed tomography were greater in men than in women,[15,16] and that the prevalence of coronary calcification in postmenopausal women was lower in estrogen users than in non-users.[17,18] The underlying mechanisms, however, remain unknown. In the present study we could show only that serum calcium level was not related to the sex difference or estrogen effect on arterial calcification. The effects were independent of atherosclerosis because 2 week administration of vitamin D did not induce any atherosclerotic change in the artery (data not shown). Because vascular smooth muscle cells play a role in calcification[22,23] and estrogen acts on vascular smooth muscle cells,[14,19] the inhibition of calcification by estrogen might be through the effects on vascular smooth muscle cells. The contribution of androgen to the sex difference was not examined in the present study. Because estrogen explains a large part of the sex difference in atherosclerosis,[10,11,14] estrogen may be more important than androgen in the sex difference in arterial calcification by analogy. The effects of gonadectomy and testosterone replacement should be examined to determine the exact role of androgen.

Recent studies have demonstrated that common factors regulate bone metabolism and vascular calcification,[22,23] as is the case with estrogen; estrogen inhibits bone resorption[24] and arterial calcification (our present results). In mice deficient in osteoprotegerin,[25] matrix Gla protein[26] and klotho,[27] both arterial calcification and abnormal bone metabolism such as osteoporosis developed. Of these factors, osteoprotegerin is reported to be regulated by estrogen.[28–30] Serum level of osteoprotegerin correlated with endogenous estrogen level[28] and was increased by estrogen replacement[29] in men. In addition, estradiol stimulates the expression of osteoprotegerin in a mouse stromal cell line via estrogen receptor-α.[30] Therefore, osteoprotegerin is one of the candidate factors that mediate the effect of estrogen on arterial calcification. Because many factors are involved in arterial calcification,[22,23] extensive examination of these factors one

by one or profiling gene expression using cDNA arrays is necessary to clarify the molecular mechanism responsible for the effect of estrogen on arterial calcification.

In summary, we found that female rats were resistant to vitamin D-induced arterial calcification, and that estrogen inhibited arterial calcification in female rats. Although the mechanism is unclear, our findings add new information to the understanding of the cardioprotective action of estrogen.

Acknowledgments

The present work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan (Grant-in-Aid for Scientific Research no. 13557062, no. 13670741) and from Kanae Foundation for Life and Socio-Medical Science.

References

1 Sangiorgi G, Rumberger JA, Severson A *et al.* Arterial calcification and not lumen stenosis is highly correlated with atherosclerotic plaque burden in humans: A histologic study of 723 coronary artery segments using nondecalcifying methodology. *J Am Coll Cardiol* 1998; **31**: 126–133.
2 Budoff MJ, Georgiou D, Brody A *et al.* Ultrafast computed tomography as a diagnostic modality in the detection of coronary artery disease: A multicenter study. *Circulation* 1996; **93**: 898–904.
3 Raggi P, Callister TQ, Cooil B *et al.* Identification of patients at increased risk of first unheralded acute myocardial infarction by electron-beam computed tomography. *Circulation* 2000; **101**: 850–855.
4 Takeo S, Anan M, Fujioka K *et al.* Functional changes of aorta with massive accumulation of calcium. *Atherosclerosis* 1989; 77: 175–181.
5 Price PA, June HH, Buckley JR, Williamson MK. Osteoprotegerin inhibits artery calcification induced by warfarin and by vitamin D. *Arterioscler Thromb Vasc Biol* 2001; **21**: 1610–1616.
6 Goodman WG, Goldin J, Kuizon BD *et al.* Coronary-artery calcification in young adults with end-stage renal disease who are undergoing dialysis. *N Engl J Med* 2000; **342**: 1478–1483.
7 Raggi P, Boulay A, Chasan-Taber S *et al.* Cardiac calcification in adult hemodialysis patients. A link between end-stage renal disease and cardiovascular disease? *J Am Coll Cardiol* 2002; **39**: 695–701.
8 Everhart JE, Pettitt DJ, Knowler WC, Rose FA, Bennett PH. Medial arterial calcification and its association with mortality and complications of diabetes. *Diabetologia* 1988; **31**: 16–23.
9 Maser RE, Wolfson Jr SK, Ellis D *et al.* Cardiovascular disease and arterial calcification in insulin-dependent diabetes mellitus: interrelations and risk factor profiles. Pittsburgh Epidemiology of Diabetes Complications Study-V. *Arterioscler Thromb* 1991; **11**: 958–965.
10 Barrett-Connor E, Bush TL. Estrogen and coronary heart disease in women. *JAMA* 1991; **265**: 1861–1867.
11 Grodstein F, Stampfer MJ. Estrogen for women at varying risk of coronary disease. *Maturitas* 1998; **30**: 19–26.
12 Hulley S, Grady D, Bush T *et al.* Randomized trial of estrogen plus progestin for secondary prevention of coronary heart disease in postmenopausal women. Heart and Estrogen/progestin Replacement Study (HERS) Research Group. *JAMA* 1998; **280**: 605–613.
13 Rossouw JE, Anderson GL, Prentice RL *et al.* Risks and benefits of estrogen plus progestin in healthy postmenopausal women: Principal results From the Women's Health Initiative randomized controlled trial. *JAMA* 2002; **288**: 321–333.
14 Mendelsohn ME, Karas RH. The protective effects of estrogen on the cardiovascular system. *N Engl J Med* 1999; **340**: 1801–1811.
15 Janowitz WR, Agatston AS, Kaplan G, Viamonte Jr M. Differences in prevalence and extent of coronary artery calcium detected by ultrafast computed tomography in asymptomatic men and women. *Am J Cardiol* 1993; **72**: 247–254.
16 Devries S, Wolfkiel C, Fusman B *et al.* Influence of age and gender on the presence of coronary calcium detected by ultrafast computed tomography. *J Am Coll Cardiol* 1995; **25**: 76–82.
17 Shemesh J, Frenkel Y, Leibovitch L, Grossman E, Pines A, Motro M. Does hormone replacement therapy inhibit coronary artery calcification? *Obstet Gynecol* 1997; **89**: 989–992.
18 Christian RC, Harrington S, Edwards WD, Oberg AL, Fitzpatrick LA. Estrogen status correlates with the calcium content of coronary atherosclerotic plaques in women. *J Clin Endocrinol Metab* 2002; **87**: 1062–1067.
19 Akishita M, Ouchi Y, Miyoshi H *et al.* Estrogen inhibits cuff-induced intimal thickening of rat femoral artery: Effects on migration and proliferation of vascular smooth muscle cells. *Atherosclerosis* 1997; **130**: 1–10.
20 Ouchi Y, Akishita M, de Souza AC, Nakamura T, Orimo H. Age-related loss of bone mass and aortic/aortic valve calcification: Reevaluation of recommended dietary allowance of calcium in the elderly. *Ann NY Acad Sci* 1993; **676**: 297–307.
21 Price PA, Buckley JR, Williamson MK. The amino bisphosphonate ibandronate prevents vitamin D toxicity and inhibits vitamin D-induced calcification of arteries, cartilage, lungs and kidneys in rats. *J Nutr* 2001; **131**: 2910–2915.
22 Tintut Y, Demer LL. Recent advances in multifactorial regulation of vascular calcification. *Curr Opin Lipidol* 2001; **12**: 555–560.
23 Shioi A, Mori K, Jono S *et al.* Mechanism of atherosclerotic calcification. *Z Kardiol* 2000; **89**: 75–79.
24 Riggs BL. The mechanisms of estrogen regulation of bone resorption. *J Clin Invest* 2000; **106**: 1203–1204.
25 Bucay N, Sarosi I, Dunstan CR *et al.* Osteoprotegerin-deficient mice develop early onset osteoporosis and arterial calcification. *Genes Dev* 1998; **12**: 1260–1268.
26 Luo G, Ducy P, McKee MD *et al.* Spontaneous calcification of arteries and cartilage in mice lacking matrix GLA protein. *Nature* 1997; **386**: 78–81.
27 Kuro-oM, Matsumura Y, Aizawa H *et al.* Mutation of the mouse klotho gene leads to a syndrome resembling ageing. *Nature* 1997; **390**: 45–51.
28 Szulc P, Hofbauer LC, Heufelder AE, Roth S, Delmas PD. Osteoprotegerin serum levels in men: Correlation with age, estrogen, and testosterone status. *J Clin Endocrinol Metab* 2001; **86**: 3162–3165.
29 Khosla S, Atkinson EJ, Dunstan CR, O'Fallon WM. Effect of estrogen versus testosterone on circulating osteoprotegerin and other cytokine levels in normal elderly men. *J Clin Endocrinol Metab* 2002; **87**: 1550–1554.
30 Saika M, Inoue D, Kido S, Matsumoto T. 17β-estradiol stimulates expression of osteoprotegerin by a mouse stromal cell line, ST-2, via estrogen receptor-alpha. *Endocrinology* 2001; **142**: 2205–2212.

ELSEVIER

Sleep Medicine 4 (2003) 403–407

SLEEP MEDICINE

www.elsevier.com/locate/sleep

Original article

Oxygen administration improves the serum level of nitric oxide metabolites in patients with obstructive sleep apnea syndrome

Shinji Teramoto[a,*], Haruki Kume[b], Takeshi Matsuse[c], Takeo Ishii[c], Akira Miyashita[c], Masahiro Akishita[d], Kenji Toba[d], Yasuyoshi Ouchi[a]

[a]*Department of Geriatric Medicine, Tokyo University Hospital, 7-3-1, Hongo Bunkyo-Ku, Tokyo 113-8655, Japan*
[b]*San-no Hospital, International University of Health and Welfare, 8-10-16, Aasaka Minato-Ku, Tokyo 107-0052, Japan*
[c]*Department of Pulmonary Medicine, Yokohama City University Medical Center, Yokohama 232-0024, Japan*
[d]*Department of Geriatric Medicine, Kyorin University, Japan*

Received 5 June 2002; received in revised form 29 November 2002; accepted 2 December 2002

Abstract

Objectives and background: Nocturnal apnea and hypoxia are implicated in the pathogenesis of pulmonary and systemic hypertension in obstructive sleep apnea syndrome (OSAS). We have hypothesized that vasodilating factors including nitric oxide (NO) are affected by nocturnal apnea and hypoxia in patients with OSAS.

Method: We examined the serum level of NO production in 24 patients with OSAS (mean age 54.2 ± 7.9 years) and 24 age-matched control subjects (53.4 ± 8.1 years) and tested the effects of oxygen administration on the production of NO in the patients.

Results: The serum level of nitrite/nitrates (NO_x), which are stable metabolites of NO, was lower in patients with OSAS than in control subjects. Administration of 1–2 l/min of oxygen during night increased the patients' NO_x level from 35.6 ± 7.3 to 57.8 ± 11.6 μM. Compressed air administration did not affect the NO_x level in the patients.

Conclusion: These results indicate that systemic NO production is impaired in OSAS patients, possibly due to nocturnal hypoxia.

Keywords: Nitric oxide; Hypoxia; Obstructive sleep apnea syndrome; Oxygen administration

1. Introduction

Obstructive sleep apnea syndrome (OSAS) is now recognized as an important sleep disorder, contributing to excessive daytime sleepiness, cardiovascular dysfunction, and the impairment of health-related quality of life [1-6]. Hypoxia, hypertension, hypoxic pulmonary vasoconstriction, pulmonary hypertension, and altered cardiovascular variability are implicated in the subsequent development of overt cardiovascular diseases, resulting in increased mortality [7,8]. However, the mechanisms underlying the causal relationship between OSAS and cardiovascular diseases are largely unknown. Nitric oxide (NO) is one of the key regulators of vascular physiology [9,10]. Abnormalities of NO productions have been implicated in the pathogenesis of pulmonary hypertension [11,12]. The concentration of NO in the exhaled air appears to be reduced in patients with pulmonary hypertension [13]. Treatment of pulmonary hypertension with NO inhalation reduces pulmonary vascular resistance in patients with pulmonary hypertension [14]. We thus speculated that the production of NO might be impaired in patients with OSAS.

The aim of the present study was to compare the serum level of NO production between OSAS and control subjects. Furthermore, to examine the relationship between NO production and nocturnal hypoxemia in patients with OSAS, we examined the effects of oxygen administration on the production of NO in patients with OSAS.

2. Methods

2.1. Subjects

All patients referred for PSG had daytime fatigue, sleepiness, and/or snoring. From April 2000 to November 2001, we invited all patients who had been referred for

* Corresponding author. Fax: +81-3-5800-8652.
E-mail address: shinjit-tky@umin.ac.jp (S. Teramoto).

1389-9457/03/$ - see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/S1389-9457(03)00102-3

a diagnostic PSG to participate in this study. Once consent had been received, patients underwent a diagnostic polysomnography (PSG).

Twenty-four patients with OSAS (19 men, 5 women, mean (±SD) aged 56 ± 4 (range 35–66)) and 24 age-matched controls (20 men; 4 women, mean (±SD) aged 53 ± 4 (range 30–68)) were studied. OSAS was defined as the presence on polysomnography of >10 obstructive sleep apneas or hypopneas per hour of sleep in association with a history of snoring and excessive daytime sleepiness. None of the subjects consumed alcohol on a regular basis or took hypnotics, sedatives, analgesics, or medications with known effects on sleep and ventilation. None of the subjects had evidence of chronic obstructive pulmonary diseases or other chronic lung disease. Four of 24 patients with OSAS were taking calcium channel blockers, compared to three of 24 control subjects. No other antihypertensive medications were prescribed for the participants in this study. We found no evidence of cardiovascular disease likely to affect pulmonary hemodynamics. Current and ex-smokers were excluded from this study because endothelial function may be affected by smoking. The demographic data relevant to the study are shown in Table 1. Because we measured the serum level of nitrite/nitrate (NO_x), we avoided the major confounding factors of NO_x measurements. All participants avoided foods rich in nitrites/nitrates, such as cured meat, for a week, and none took drugs known to increase NO_x levels (angitensin-converting enzyme (ACE) inhibitors and nitroglycerin).

Spirometry was performed using standard techniques [15] (CHESTAC-5v, Chest Co., Tokyo, Japan). Forced expiratory maneuvers were performed in triplicate, and the best effort was analyzed. Measurements were made on forced vital capacity (FVC) and forced expiratory volume in 1 s (FEV_1). All testing was performed with the patient in a seated position. The pulmonary function test data were expressed as a percentage of predicted normal values.

Table 1
Demographic and anthropometric data

	OSAS	CTRL
Number of subjects	24	24
Male: female	19:5	20:4
Age (years)	54.2 ± 3.6	53.2 ± 3.6
Height (cm)	164 ± 6	162 ± 5
Weight (kg)	78 ± 6	75 ± 6
BMI (wt/(ht)^2)	29.0 ± 1.6	28.6 ± 1.7
FVC (l)	2.82 ± 0.26	2.79 ± 0.22
FEV_1(l)	2.42 ± 0.12	2.38 ± 0.11
FEV_1/FVC (%)	85.8 ± 5.6	85.3 ± 5.1
PaO_2 (mmHg)	68.2 ± 2.1	70.2 ± 1.8
$PaCO_2$ (mmHg)	42.2 ± 1.3	41.8 ± 1.2
SBP (mmHg)	138 ± 4.8	132 ± 4.6
DBP (mmHg)	74 ± 2.4	73 ± 2.2

Data were presented as mean ± SD.

OSAS, obstructive sleep apnea syndrome; CTRL, control subjects without OSAS; BMI, body mass index,; FVC forced vital capacity (l); FEV_1, forced expiratory volume in 1 s (l); PaO_2, arterial pressure of oxygen; $PaCO_2$, arterial pressure of carbon dioxide; SBP, systolic blood pressure; DBP, diastolic blood pressure.

3. Sleep study

All subjects were admitted for two or more consecutive nights for polysomnographic study. Polysomnography consisted of 8 h of overnight monitoring using a standard technique [16]. Respiratory effort was measured by respiratory inductance plethymography (Respitrace Corp., USA), and airflow at the nose and mouth was measured with thermistors. Surface electrodes were applied to obtain an electroencephalogram (EEG), electrooculogram (EOG), electrocardiogram, and a record of heart rate. Arterial oxygen saturation (SaO_2) was recorded with a pulse oximeter (502-P, Criticare System Inc., Centrouis, MO, USA). A polygraph was used to record data on both the paper of a 6-channel chart recorder (Nihonkoden, Tokyo, Japan) and a floppy disk via an IBM-compatible personal computer data acquisition system (NEC 9801, NEC, Tokyo, Japan). In subjects who slept for less than 6 h, as determined by EEG and EOG, repeat sleep studies were performed to assess whether poor sleep led to a missed diagnosis or inaccurate estimation of disease severity. Apnea was defined as the cessation of oronasal airflow for more than 10 s. Oxyhemoglobin desaturation was not a criterion for scoring apneas. The hypopneas are further defined by flow reduction and 2% desaturation. Flow reduction was defined as a reduction of 50% or more for at least 10 s in the oronasal flow in relation to prevailing values during preceding normal breathing. In this study, sleep apnea syndrome was determined by apnea + hypopnea index (AHI) values greater than 10/h. We did not measure body position or leg movement in the study.

After nocturnal oxygen administration (1–2 l/min) via nasal prong for 1 week, sleep studies were repeated to assess the effects of oxygen supplement on the severity of nocturnal apneas and arterial oxygen desaturation in OSAS patients. For the control arm of the study, the same group received nocturnal administration of compressed air via nasal prong for a week, and the sleep study was repeated to assess the effects of air administration on the NO_x levels, severity of nocturnal apneas, and arterial oxygen desaturation in these patients. The flow rate of oxygen/air administration was determined by the nadir SaO_2; 2 l/min of oxygen/air was administered when the nadir SaO_2 was smaller than 80%. Oxygen and air were randomly administered using a crossover protocol with a 1 week washout period.

3.1. Serum nitrite/nitrate measurement

Peripheral blood samples were obtained from the OSAS patients at 8.00 AM and 8:00 PM, before and after a week of

oxygen administration. Blood samples were collected in ice-cooled tubes containing lithium–heparin. Samples were deproteinized before analysis with 4% $ZnSO_4$. Serum nitrite/nitrate (NO_x) concentrations were determined using an analyzer employing the Griess method as previously described [17]. Data presented in the tables were obtained by means of addition of the NO_2^- plus NO_3^- concentrations, expressed in μM. In most samples, NO_3^- accounted for more than 90% of the total value.

3.2. Statistical analysis

The Mann–Whitney nonparametric test was used to compare the variables of demographic data and pulmonary function data in OSAS patients and control subjects. An analysis of variance with one fixed factor with repeated measures was used to compare the variables in baseline, air, and oxygen in the two subject groups. The association between serum NO_x levels and other variables was assessed separately. The analyses were performed by a software package using Stat View 4.0 (Abacus Concepts, Inc., Berkeley, CA, USA). The data are presented as mean ± SD. $p < 0.05$ was considered to be statistically significant.

4. Results

In the current study, all the participants in both the OSAS group (body mass index (BMI) = 28.8 ± 2.0) and the control group (BMI = 28.4 ± 3.3) were obese. The control subjects were matched for age and BMI. spirometric indices, and variables of arterial blood gas were within normal range in all subjects. Anthropometric and pulmonary function data are shown in Table 1. The control subjects were also matched for parameters of pulmonary function testing and blood gas analysis. In the OSAS group, all 20 patients had moderate to severe obstructive sleep apnea (obstructive or mixed apneas/hypopneas per hour of sleep were greater than 10) and the mean AHI was 38.6 ± 4.8. No member of the control group had more than five apneas/hypopneas per hour (AHI was less than 5) and mean AHI was 1.8 ± 0.8 (Table 2). Although the baseline value of SaO_2 in patients with OSAS was not different from that in the control subjects, the nadir SaO_2 values were considerably lower in OSAS patients than in control subjects ($p < 0.01$). While the number of apneas among OSAS patients was not significantly reduced by oxygen administration, the 4% arterial oxygen desaturations from baseline SaO_2 was markedly reduced (Table 2). The nadir SaO_2 was improved by the oxygen supplementation but not by air administration. In control subjects, 1–2 l/min of oxygen administration did not affect the number of apneas or the nadir SaO_2 (Table 2).

The serum level of NO_x in OSAS patients was lower than that in control subjects (Figure 1) (Table 3). Oxygen administration significantly increased the serum NO_x levels in every patient, but they did not reach the normal levels of the control subjects. Air administration had no effect on the NO_x levels in the patients. Oxygen administration did not affect the serum NO_x levels among control subjects (Table 3).

We examined the relationship between the NO_x levels and the following parameters: AHI, nadir SaO_2, systolic blood pressure, diastolic blood pressure, and arterial oxygen. There were significant relationships between NO_x levels, nadir SaO_2, and the 4% oxygen desaturations (Table 4). However, AHI and systolic/diastolic blood pressures were not correlated with NO_x levels (Table 4).

5. Discussion

The present study demonstrates that the serum levels of nitrite/nitrate (NO_x), which are stable metabolites of NO,

Table 2
Effects of the supplementation of oxygen or compressed air on the numbers of apneas and arterial oxygen desaturation in OSAS patients and control subjects

Supplementation	OSAS			CTRL		
	Non	Air	Oxygen	Non	Air	Oxygen
Number of subjects	24			24		
AHI (/h)	39. ± 5*	36 ± 6*	36 ± 6*	2 ± 1	2 ± 2	2 ± 2
Baseline SaO_2 (%)	95 ± 2	95 ± 2	96 ± 1	95 ± 2	97 ± 2	96 ± 2
Nadir SaO_2 (%)	70 ± 8*	73 ± 4*	90 ± 3#	93 ± 2	95 ± 2	93 ± 1
N of 4% desaturation	264 ± 5*	246 ± 7*	28 ± 6*	4 ± 2	2 ± 2	3 ± 2

Data were presented as mean ± SD.

OSAS, obstructive sleep apnea syndrome; CTRL, control subjects without OSAS; air: 1–2 ml/min of compressed air was administered during night via nasal prong in for a week.

Oxygen: 1 week oxygen administration (1–2 l/min) during night via nasal prong. AHI, apnea and hypopnea index; baseline SaO_2, stable value of SaO_2 at supine position before sleep (%); nadir SaO_2, the lowest value of SaO_2 during night (%); N of 4% desaturation, number of 4% of arterial oxygen desarutation from baseline value of SaO_2.

*$p < 0.01$ compared with the same value of CTRL.

#$p < 0.01$ compared with the same value without oxygen supplementation.

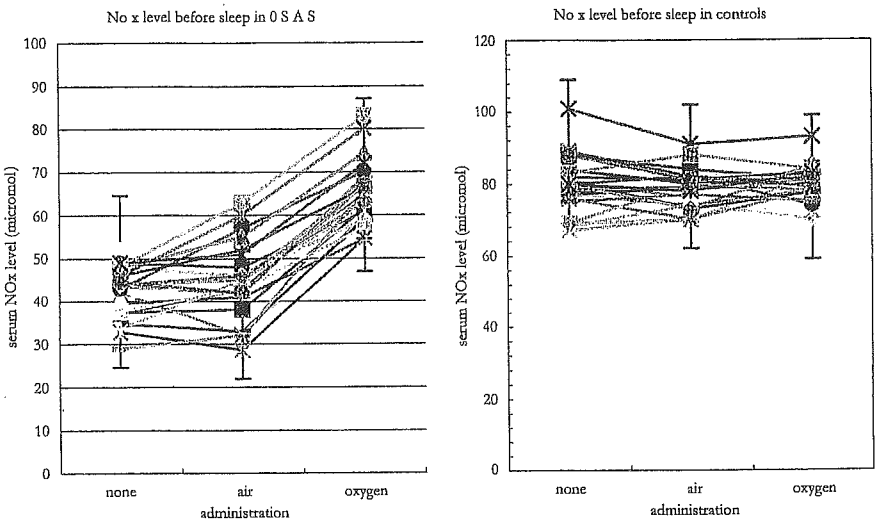

Fig. 1. Serum NO_x levels before sleep in patients with OSAS and in obese controls without OSAS.

were smaller in OSAS patients than in control subjects. It has recently been reported that the early morning serum NO_x levels were significantly lower in OSAS subjects than in control subjects (OSAS = 38.9 μM, control subjects = 63.1 μM) [18]. Schulz and coworkers have reported that NO_x levels were 21.7 μM in OSA patients, compared with 42.6 μM in healthy volunteers and 36.7 μM in control patients without OSA but with a similar spectrum of co-morbidity [19]. Our data support these previous observations.

We report that there is a significant negative correlation between serum nitrites/nitrates and the following parameters: AHI, oxygen desaturation time, and systolic blood pressure. Examination of the relationship between the NO_x level and AHI, nadir SaO_2, systolic blood pressure, diastolic blood pressure, and arterial oxygen desaturation indicates that the level of NO_x is significantly correlated with the number and amount of oxygen desaturations but not with AHI and blood pressure. Repeated episodes of nocturnal hypoxemia may be due to the impaired production of NO. Comparing the effects of nocturnal oxygen supplementation on apneas and NO_x production in OSAS patients with the effects of compressed air supplementation revealed that oxygen (but not air) increased the NO_x level but did not affect the apneas. It is reasonable to assume that repeated episodes of nocturnal hypoxemia are a mechanism of the impaired NO production in patients with OSAS. Because oxygen is a cosubstrate of NO synthase (NOS), OSAS-related nocturnal desaturations might result in depressed synthesis of NO.

The current results are at least supported in part by recent evidence that nasal continuous positive airway pressure (nCPAP) reverses the suppressed NO in OSAS patients [18,19]. However, the nCPAP might reverse both apneas and hypoxemia; there is a possibility that nocturnal apneas themselves are involved in the impaired NO production. Because oxygen supplementation increased the NO production but did not totally reverse the NO_x levels in the current study, we must consider the possibility that nocturnal apnea itself, and other factors, may contribute to endothelium dysfunction in OSAS patients. It has been demonstrated that NOS inhibitors are elevated in OSAS patients and thus might also contribute to lowered NO_x levels [20]. The nitrite/nitrate (NO_x) levels might be influenced by a variety of confounding factors, such as arterial hypertension, cigarette smoking, hypercholesterolemia,

Table 3
Serum concentration of NO_x before and after sleep studies

Supplementation	OSAS			CTRL		
	Non	Air	Oxygen	Non	Air	Oxygen
NO_x before sleep (μM)	43.7 ± 6.2*	44.7 ± 6.4*	65.8 ± 6.4#	79.6 ± 7.3	78.6 ± 5.3	80.6 ± 4.3
NO_x after sleep (μM)	35.6 ± 5.3*	37.6 ± 5.9*	58.8 ± 7.3#	72.6 ± 4.3	75.6 ± 5.2	73.2 ± 4.1

Data were presented as mean ± SD.
Air: 1–2 ml/min of compressed air was administered during night via nasal prong for a week.
Oxygen: 1 week oxygen administration (1–2 l/min) during night via nasal prong.
*$p < 0.01$ compared with the same value of CTRL.
#$p < 0.01$ compared with the same value without air/oxygen supplementation.

Table 4
Relationships between the serum level of NO_x and other variables

	p	r
AHI (/h)	(–)	
Baseline SaO_2 (%)	(–)	
Nadir SaO_2 (%)	0.05	−357
N of 4% desaturation	0.01	−401
Systolic BP	(–)	
Diastolic BP	(–)	

BP, blood pressure.

and diabetes mellitus. Thus, we should further determine by direct comparison the effects of CPAP and O_2 on the NO_x levels in patients.

It has been reported, using the measurements of brachial artery diameter under baseline conditions, during reactive hyperemia and after sublingual administration of nitroglycerin (an endothelium-independent vasodilator), that patients with OSAS have an impairment of resistance-vessel endothelium-dependent vasodilation [21]. Although the reduced levels of NO_x were identified in patients with OSAS, the functional impairment of endothelium-dependent vasodilators should be further examined. NO, being one of the mediators, may be involved in the hemodynamic regulation and long-term vascular remodeling in OSAS patients.

In conclusion, these results indicate that systemic NO production is impaired in OSAS patients, possibly due to nocturnal hypoxia.

Acknowledgements

This study was supported by the 21st Medical frontier research grant from the Ministry of Health and Welfare and Japan Arteriosclerosis Prevention Fund.

References

[1] Guilleminault C, Tilkian A, Dement WC. The sleep apnea syndromes. Annu Rev Med 1976;27:465–84.
[2] Young T, Palta M, Dempsey J, et al. The occurrence of sleep-disordered breathing among middle-aged adults. N Engl J Med 1993; 328:1230–5.
[3] Teramoto S, Ohga E, Ouchi Y. Obstructive sleep apnoea. Lancet 1999;354:1213–4.
[4] Jenkinson C, Davies RJ, Mullins R, et al. Comparison of therapeutic and subtherapeutic nasal continuous positive airway pressure for obstructive sleep apnoea: a randomised prospective parallel trial. Lancet 1999;353:2100–5.
[5] Teramoto S, Matsuse T, Ouchi Y. Does the altered cardiovascular variability associated with obstructive sleep apnea contribute to development of cardiovascular disease in patients with obstructive sleep apnea syndrome? Circulation 1999;100:e136–7.
[6] Teramoto S, Matsuse T, Fukuchi Y. Clinical significance of nocturnal oximeter monitoring for detection of sleep apnea syndrome in the elderly. Sleep Med 2002;3:17–21.
[7] Ziegler MG, Nelesen R, Mills P, et al. Sleep apnea, norepinephrine-release rate, and daytime hypertension. Sleep 1997;20:224–31.
[8] Teramoto S, Kume H, Matsuse T, et al. The risk of future cardiovascular diseases in the patients with OSAS is dependently or independently associated with obstructive sleep apnoea. Eur Respir J 2001;17:573–4.
[9] Hoover DB, Chang Y, Hancock JC, et al. Actions of tachykinins within the heart and their relevance to cardiovascular disease. Jpn J Pharmacol 2000;84:367–73.
[10] Ozaki K, Yamamoto T, Ishibashi Y, et al. Regulation of endothelial nitric oxide synthase and endothelin-1 expression by fluvastatin in human vascular endothelial cells. Jpn J Pharmacol 2001;85:147–54.
[11] Giaid A, Saleh D. Reduced expression of endothelial nitric oxide synthase in the lungs of patients with pulmonary hypertension. N Engl J Med 1995;333:214–21.
[12] Stamler JS, Loh E, Roddy MA, et al. Nitric oxide regulates basal systemic and pulmonary vascular resistance in healthy humans. Circulation 1994;89:2035–40.
[13] Kharitonov SA, Cailes JB, Black CM, et al. Decreased nitric oxide in the exhaled air of patients with systemic sclerosis with pulmonary hypertension. Thorax 1997;52:1051–5.
[14] Pepke-Zaba J, Higenbottam TW, Dinh-Xuan AT, et al. Inhaled nitric oxide as a cause of selective pulmonary vasodilatation in pulmonary hypertension. Lancet 1991;338:1173–4.
[15] Teramoto S, Fukuchi Y, Orimo H. Effects of anticholinergic drug on dyspnea and gas exchange in patients with chronic obstructive pulmonary disease. Chest 1993;103:1774–82.
[16] Teramoto S, Sudo E, Mstsuse T, et al. Impaired swallowing reflex in patients with obstructive sleep apnea syndrome. Chest 1999;116: 17–21.
[17] Watanabe T, Akishita M, Toka M, et al. Influence of sex and age on serum nitrite/nitrate concentration in healthy subjects. Clin Chim Acta 2001;301:169–79.
[18] Ip MS, Lam B, Chan LY, et al. Circulating nitric oxide is suppressed in obstructive sleep apnea and is reversed by nasal continuous positive airway pressure. Am J Respir Crit Care Med 2000;162:2166–71.
[19] Schulz R, Schmidt D, Blum A, et al. Decreased plasma levels of nitric oxide derivatives in obstructive sleep apnea: response to CPAP therapy. Thorax 2000;55:1046–51.
[20] Carlson J, Hedner J, Pettersson A. Increased plasma concentration of ADMA, a naturally occurring nitric oxide synthesis inhibitor, in OSA patients. Am J Respir Crit Care Med 1997;155:A869.
[21] Kato M, Roberts-Thomson P, Phillips BG, et al. Impairment of endothelium-dependent vasodilation of resistance vessels in patients with obstructive sleep apnea. Circulation 2000;102:2607–10.

□ ORIGINAL ARTICLE □

Effects of Oxygen Administration on the Circulating Vascular Endothelial Growth Factor (VEGF) Levels in Patients with Obstructive Sleep Apnea Syndrome

Shinji TERAMOTO, Haruki KUME*, Hiroshi YAMAMOTO, Takeo ISHII**, Akira MIYASHITA**, Takeshi MATSUSE**, Masahiro AKISHITA***, Kenji TOBA*** and Yasuyoshi OUCHI

Abstract

Objective **Repeated nocturnal hypoxia is implicated in the pathogenesis of cardiovascular complications in obstructive sleep apnea syndrome (OSAS). We hypothesized that circulating vascular endothelial growth factor (VEGF) levels are affected by nocturnal hypoxemia in patients with OSAS.**

Methods **We examined the serum VEGF levels in patients with OSAS and in control subjects. We also tested the effects of oxygen or air administration on the subjects' VEGF levels.**

Patients and Materials **Twenty-four OSAS patients (mean age 54.2±3.6 years) and 24 age-matched control subjects (53.2±3.6 years). Their serum samples were tested.**

Results **Serum VEGF levels at 8 : 00 AM were significantly higher in OSAS patients than in controls ($p<0.01$). VEGF levels decreased from 515±31 (pg/ml) to 178±16 (pg/m) ($p<0.01$) in OSAS patients whose nocturnal hypoxemia was found to be improved by administration of 2 *l*/min of oxygen during the night. However, the administration of compressed air affected neither the VEGF level nor nocturnal oxygen desaturation in OSAS patients.**

Conclusion **These results indicate that circulating VEGF levels are elevated in OSAS patients, primarily due to nocturnal hypoxemia.**

(Internal Medicine 42: 681–685, 2003)

Key words: OSAS, hypoxemia, oxygen therapy, cardiovascular complications, vascular remodeling

Introduction

Obstructive sleep apnea syndrome (OSAS) is now recognized as an important sleep disorder, contributing to excessive daytime sleepiness, cardiovascular dysfunction and the impairment of health-related quality of life (1–6). Intermittent repeated hypoxia during night, hypoxic pulmonary vasoconstriction and altered cardiovascular variability are implicated in the subsequent development of overt cardiovascular diseases, resulting in increased mortality (7, 8). There is growing evidence that patients with OSAS have an increased risk of having cardiovascular complications, such as hypertension, cardiac arrhythmia (9), myocardial infarction (10), pulmonary hypertension (11), and stroke (12). Vascular endothelial growth factor (VEGF) is a potent angiogenic factor whose expression is induced by hypoxia (13, 14). VEGF has detectable levels in circulation and its expression is highly regulated by oxygen tension. We therefore hypothesized that serum VEGF levels are elevated in OSAS patients.

The aim of the present study was to compare the serum level of VEGF between OSAS and control subjects. Additionally, in order to define the causal relationship between circulating VEGF levels and nocturnal hypoxemia in OSAS patients, we examined the effects of oxygen administration on serum VEGF levels in patients with OSAS.

Methods

Subjects

Twenty-four patients with OSAS [19 men : 5 women, mean (±SD) aged 54.2±3.6 (range 35–66)] and 24 age-

From the Department of Geriatric Medicine, *the Department of Urology, The University of Tokyo Hospital, Tokyo, **the Department of Pulmonary Medicine, Yokohama City University Medical Center, Yokohama and ***Department of Geriatric Medicine, Kyorin University, Tokyo

Received for publication July 23, 2002; Accepted for publication April 9, 2003

Reprint requests should be addressed to Dr. Shinji Teramoto, the Department of Geriatric Medicine, The University of Tokyo Hospital, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655

matched controls [20 men : 4 women, mean (±SD) aged 53.2±3.6 (range 30–68)] were studied. OSAS was defined as the presence of >10 obstructive sleep apneas or mixed apneas per hour of sleep on polysomnography in association with snoring and excessive daytime sleepiness. No subjects consumed alcohol on a regular basis, nor did they take hypnotics, sedatives, analgesics, or other medications with known effects on sleep and ventilation. No subjects had evidence of chronic obstructive pulmonary diseases or other chronic lung disease, and no evidence was found of any cardiovascular disease likely to affect pulmonary hemodynamics. Current and ex-smokers were excluded from this study, since endothelial function may be affected by smoking. The demographic data relevant to the study are shown in Table 1. Informed consent was obtained from all subjects. The study protocol was approved by the committee on ethics and the Institutional Review Board of Tokyo University Hospital.

Spirometry and expiratory flow were measured using a spirometer (CHESTAC-5v, Chest Co., Tokyo, Japan). Forced vital capacity (FVC) and forced expiratory volume in one second (FEV_1) were measured. For spirometric indices, we selected the best of the three maximal flow-volume curves with the best being defined as the curve with the highest sum of FVC and FEV_1 (15).

Sleep study

For polysomnographic study, all subjects were admitted to our hospitals for two or more consecutive nights. Polysomnography consisted of 8 hours of overnight monitoring using the standard technique (16). Respiratory effort was measured by respiratory inductance plethymography (Respitrace Corp., Ardsley, NY, USA), and thermistors were used to measure airflow at the nose and mouth. Surface electrodes were applied to obtain an electroencephalogram, electrooculogram [EOG], electrocardiogram, and a record of heart rate. Arterial oxygen saturation (SaO_2) was recorded with a pulse oximeter (502-P, Criticare Systems Inc., Centrais, MO, USA). A polygraph was used to record data on both the paper of a 6-channel chart recorder (Nihonkoden Co., Tokyo, Japan) and a floppy disk via an IBM-compatible personal computer data acquisition system (NEC 9801, NEC, Tokyo, Japan). In subjects who slept for less than 6 hours as determined by EEG and EOG, repeat sleep studies were performed to assess whether poor sleep led to a missed diagnosis or an inaccurate estimation of disease severity. Apnea was defined as the cessation of oronasal airflow for more than 10 seconds, and hypopnea as a reduction of 50% or more in the oronasal flow in relation to the prevailing values during preceding normal breathing, with the reduction lasting for at least 10 seconds. For determinant of the role of apenas/hypopneas wihtout hypoxemia on the serum levels of VEGF, oxyhemoglobin desaturation was not a criterion for scoring either apnea or hypopnea. For the purpose of this study, sleep apnea syndrome (SAS) was considered to be determined by apnea+hypopnea index (AHI) values greater than 10/hour.

After one week of oxygen administration (2 *l*/min) during the night via nasal prong, sleep studies were repeated to assess the effects of oxygen supplementation on the severity of apneas and arterial oxygen desaturation during the night in patients with OSAS. As a control study, the same group of OSAS patients also received compressed air for one week during the night via nasal prong. Sleep studies were then repeated to assess the effects of air administration on the VEGF levels, severity of apneas and arterial oxygen desaturation during the night in patients with OSAS. Oxygen and air were randomly administered using a cross-over protocol with a 1-week washout period.

Quantification of VEGF concentration in serum

Peripheral blood samples were obtained from all OSAS patients at 8:00 AM and 8:00 PM on the day prior to administration of oxygen or compressed air, and on the day after 1-week of oxygen or compressed air administration. The samples were immediately centrifuged for 10 minutes at 3,000 rpm. The supernatant fluid was stored in a refrigerator at –70°C for later analysis. Serum levels of VEGF were determined by a commercially available ELISA test (R&D Systems, Wiesbaden, Germany). This assay, which measures free, unbound VEGF, is calibrated against a highly purified recombinant human $VEGF_{165}$, the major and most potent isoform of VEGF. The VEGF assay had a minimum sensitivity of 9 pg/ml, with intra-assay coefficients of variation of 6.7% and 4.5% for concentrations of 54 and 235 pg/ml, respectively. All VEGF measurements were carried out on the same day after completion of all sleep studies.

Statistical analysis

The Mann-Whitney nonparametric test was used to compare the variables of demographic data and pulmonary function data in both OSAS patients and control subjects. An analysis of variance with 1 fixed factor with repeated measures was used to compare the variables baseline, air and oxygen and 2 subject groups. The associations of serum VEGF levels with other variables were tested using linear regression analysis. All analyses were performed by a software package using Stat View 5.0 (Abacus Concepts, Inc., Berkeley, CA, USA). The data are presented as mean±SD. $p<0.05$ was considered to be statistically significant.

Results

In the current study, all participants were obese, both in the OSAS group [body mass index (BMI)=29.0±1.6 (kg/m^2)] and in the control (CTRL) group [BMI=28.6±1.7 (kg/m^2)]. Spirometric indices were within the normal range in all subjects. Anthropometric and pulmonary function data are shown in Table 1. In the OSAS group, all 24 OSAS patients had moderate to severe obstructive sleep apnea (i.e., obstructive or mixed apneas/hypopneas per hour of sleep were greater than 10) and their mean AHI was 38.6±4.8. In the CTRL group, no subject showed more than 5 apneas/

Table 1. Demographic and Anthropometric Data

	OSAS	CTRL
Number of subjects	24	24
Male : Female	19 : 05	20 : 04
Age (years old)	54.2±3.6	53.2±3.6
Height (cm)	164±6	162±5
Weight (kg)	78±6	75±6
BMI (kg/m^2)	29.0±1.6	28.6±1.7
FVC (*l*)	2.82±0.26	2.79±0.22
FEV_1(*l*)	2.42±0.12	2.38±0.11
FEV_1/FVC (%)	85.8±5.6	85.3±5.1
PaO_2 (mmHg)	68.2±2.1	70.2±1.8
$PaCO_2$ (mmHg)	42.2±1.3	41.8±1.2
SBP (mmHg)	138±4.8	132±4.6
DBP (mmHg)	74±2.4	73±2.2

Data were presented as mean±SD. OSAS: obstructive sleep apnea syndrome, CTRL: control subjects without OSAS, BMI: body mass index, FVC: forced vital capacity (*l*), FEV_1: forced expiratory volume in one second (*l*), PaO_2: arterial pressure of oxygen, $PaCO_2$: arterial pressure of carbon dioxide, SBP: systolic blood pressure, DBP: diastolic blood pressure.

hypopneas per hour, and their AHI was 1.8±0.8 (Table 2). Although the baseline value of SaO_2 in patients with OSAS was nearly identical to that in the control subjects, the nadir SaO_2 values were considerably lower in patients with OSAS than in control subjects ($p<0.01$). While the occurrence of apnea during sleep in patients with OSAS was not significantly reduced by the administration of oxygen, the rate of 4% arterial oxygen desaturation from baseline SaO_2 in our patients was markedly reduced by oxygen administration (Table 2). Both nadir SaO_2 and mean SpO_2 during night were improved by oxygen supplementation, but not by the administration of compressed air. In control subjects without OSAS, oxygen administration (2 *l*/min) did not affect the number of apneas or the nadir SaO_2 (Table 2).

The serum VEGF level (515±31 pg/ml) after sleep was higher ($p<0.01$) in OSAS patients than in control subjects (152±13 pg/ml). The value before sleep, taken at 8:00 PM, was also higher in OSAS patients than in control subjects ($p<0.01$) (Table 3). After oxygen administration, VEGF levels in OSAS patients both before and after sleep decreased significantly. However, the administration of air had no effect on their serum VEGF levels. In control subjects, the administration of neither oxygen nor air affected the serum

Table 2. Effects of the Supplementation of Oxygen or Compressed Air on the Numbers of Apneas and Arterial Oxygen Desaturation in OSAS Patients and Control Subjects

Supplementation	OSAS			CTRL		
	Non	Air	Oxygen	Non	Air	Oxygen
Number of subjects	24	24	24	24	24	24
AHI (/hour)	39±5*	36±6*	35±6*	2±1	2±2	2±2
Baseline SaO_2 (%)	95±2	96±1	95±2	95±2	96±2	97±2
Nadir SaO_2 (%)	70±8*	73±4#	90±3	93±2	93±2	95±1
N of 4% desaturation	264±5*	246±7*#	28±6*	4±2	3±2	2±2
Mean SaO_2 (%)	89±2*	90±1*#	93±1	94±1	94±2	95±1

Data were presented as mean±SD. OSAS: obstructive sleep apnea syndrome, CTRL: control subjects without OSAS, Air: 1–2 ml/min of compressed air was administered during the night via nasal prong for a week. Oxygen: a week oxygen administration (2 *l*/min) during the night via nasal prong. AHI: apnea and hypopnea index, Baseline SaO_2: stable value of SaO_2 at supine position before sleep (%), Nadir SaO_2: the lowest value of SaO_2 during night (%), N of 4% desaturation: number of 4% of arterial oxygen desaturation from baseline value of SaO_2, Mean SpO_2: the mean value of SaO_2 during night (%). *$p<0.01$ compared with the same value of CTRL, #$p<0.01$ compared with the same value without oxygen supplementation.

Table 3. Serum Levels of VEGF before and after Sleep Studies

Supplementation	OSAS			CTRL		
	Non	Air	Oxygen	Non	Air	Oxygen
VEGF before sleep (μM)	457±26*	469±29*	165±12#	161±23	158±13	145±16
VEGF after sleep (μM)	515±31*	526±34*	178±16#	152±13	165±22	150±14

Data were presented as mean±SD. Air: 2 ml/min of compressed air was administered during the night via nasal prong for a week. Oxygen: one week of oxygen administration (2 *l*/min) during the night via nasal prong. *$p<0.01$ compared with the same value of CTRL, #$p<0.01$ compared with the same value without air/oxygen administration.