

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

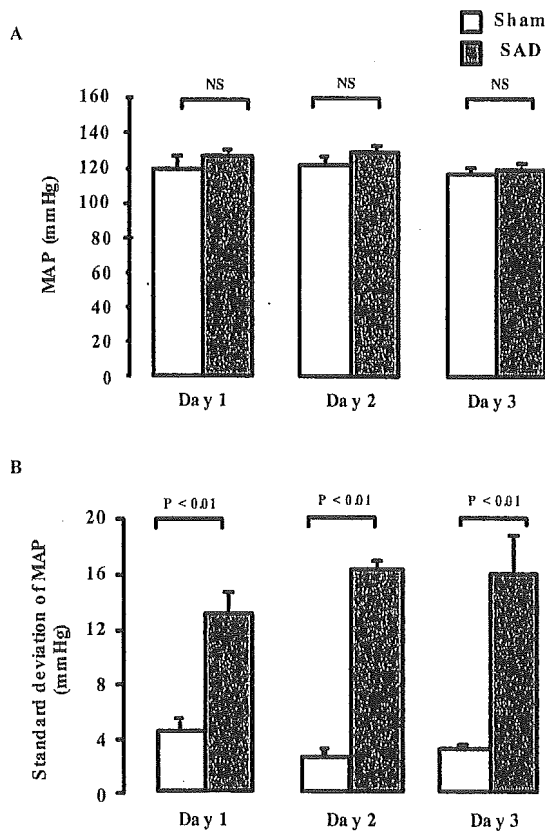


Fig. 3. Average level of mean arterial pressure (MAP) (A) and standard deviation of MAP (B) from day 1 to day 3 after sinoaortic-denervation (SAD) or sham operation (Sham) in rats. Each bar represents the mean \pm SEM (n=5 for each group).

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

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

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

Values are expressed as mean \pm 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 \pm 0.15	0.87 \pm 0.05
Acetylcholine		
Maximum relaxation (% of precontraction)	90.6 \pm 3.0	66.6 \pm 2.2*
pD_2 (–log mol/l)	7.21 \pm 0.26	6.44 \pm 0.16*
Sodium nitroprusside		
Maximum relaxation (% of precontraction)	98.3 \pm 1.3	95.8 \pm 1.6
pD_2 (–log mol/l)	6.94 \pm 0.12	6.69 \pm 0.10

Values are expressed as mean \pm 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.

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

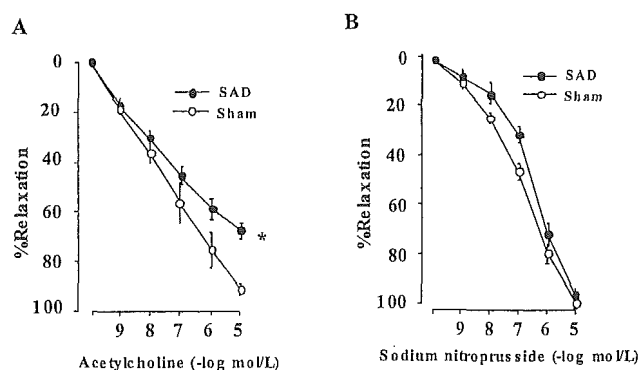


Fig. 4. Endothelium-dependent relaxation response to acetylcholine (A) and endothelium-independent relaxation response to sodium nitroprusside (B) in isolated aortic rings of sinoaortic-denervated rats (SAD) and sham-operated rats (Sham). Data are expressed as the percent relaxation of contraction evoked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

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 μ mol/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, $11.8 \pm 1.0\%$; sham-operated group, $8.2 \pm 1.0\%$; $n=6$, $p<0.05$).

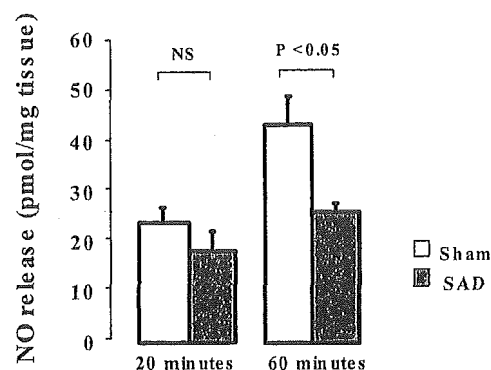


Fig. 5. Acetylcholine (1 μ mol/l)-induced NO production by isolated aortic rings derived from sinoaortic-denervated rats (SAD) and sham-operated rats (Sham). Each bar represents the mean \pm SEM ($n=5-7$ for each group).

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

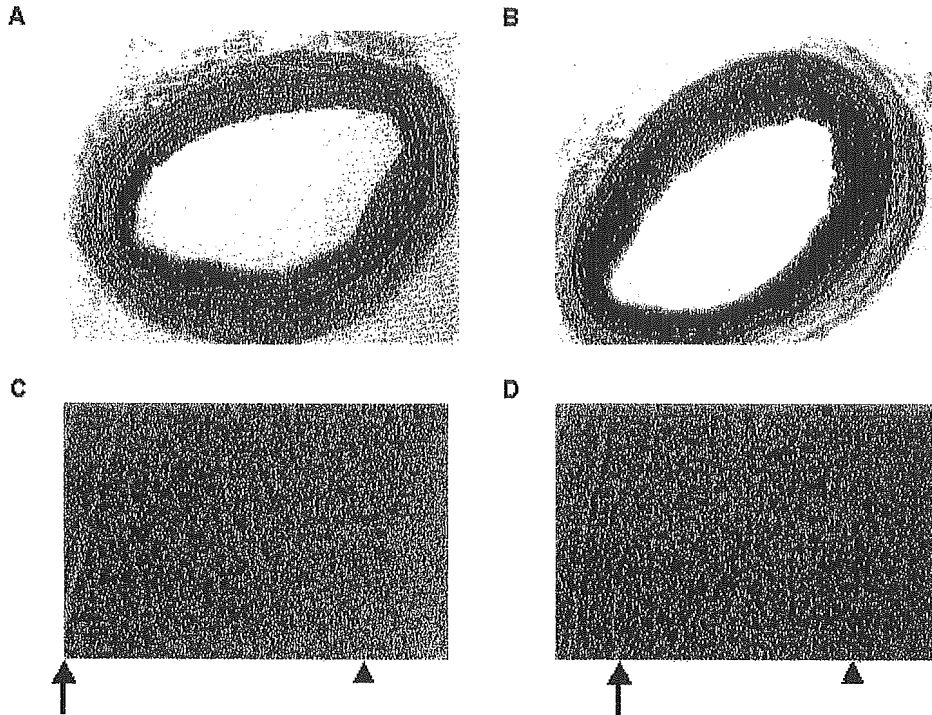


Fig. 6. Representative histological sections of common carotid arteries 14 days after balloon injury from a sham-operated rat (A, C) and a sinoaortic-denervated rat (SAD) (B, D). Elastica van Gieson staining (A, B) and immunohistochemical staining using anti-proliferating cell nuclear antigen (C, D).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

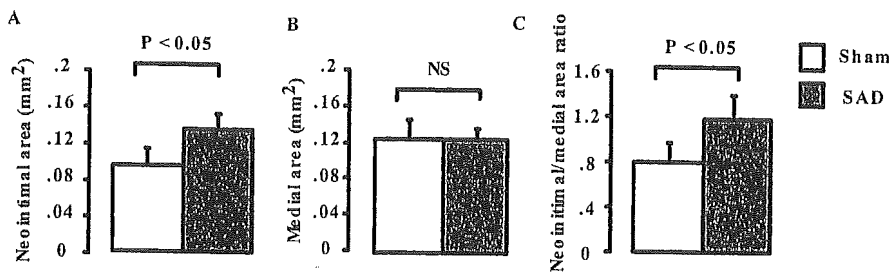


Fig. 7. A and B show cross-sectional areas of the neointima and media, respectively, of carotid arteries 14 days after balloon injury from sinoaortic-denervated rats (SAD) and sham-operated rats (Sham). C shows the neointimal-to-medial area ratio of carotid arteries from the same two groups. Each bar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

um-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.

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ORIGINAL ARTICLE

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

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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)D₃ 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)D₃ 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.

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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)D₃ (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)D₃ 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)D₃ 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)D₃ (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)D₃ 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)D₃ and between 8-week-old and 30-week-old rats. Calcium content in the aorta was increased by vitamin 1α(OH)D₃ 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)D₃ 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).

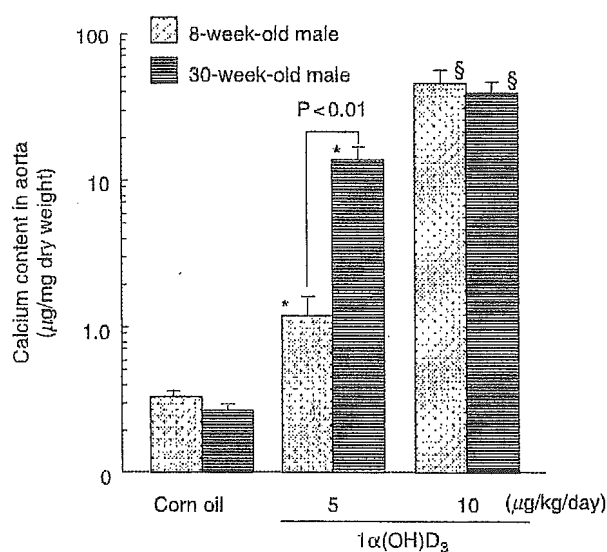


Figure 1 Influence of age and vitamin D dosage on arterial calcification in male rats. Vehicle or $1\alpha(\text{OH})\text{D}_3$ (5 or 10 $\mu\text{g}/\text{kg}$ /per ay) was given to 8-week-old and 30-week-old male rats for 2 weeks. Calcium content in the aorta was then measured and normalized by dry weight. * $P < 0.01$ vs corn oil, § $P < 0.01$ vs corn oil and 5 $\mu\text{g}/\text{kg}$ per day $1\alpha(\text{OH})\text{D}_3$. $n = 5$ 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 $\mu\text{g}/\text{kg}$ per day $1\alpha(\text{OH})\text{D}_3$ for 2 weeks. Serum estradiol concentration was 38 ± 7 pmol/L for vehicle and 35 ± 6 pmol/L for $1\alpha(\text{OH})\text{D}_3$ in male rats, and 128 ± 31 pmol/L for vehicle and 120 ± 35 pmol/L for $1\alpha(\text{OH})\text{D}_3$ in female rats ($P < 0.05$ vs male). Serum calcium concentration was not different between male and female rats although $1\alpha(\text{OH})\text{D}_3$ raised serum calcium concentration significantly in each sex; 9.8 ± 0.2 mg/dL for vehicle and 13.2 ± 0.2 mg/dL for $1\alpha(\text{OH})\text{D}_3$ in male rats ($P < 0.01$); 10.3 ± 0.1 mg/dL for vehicle and 14.4 ± 0.3 mg/dL for $1\alpha(\text{OH})\text{D}_3$ 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 $\mu\text{g}/\text{kg}$ per day $1\alpha(\text{OH})\text{D}_3$ 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.

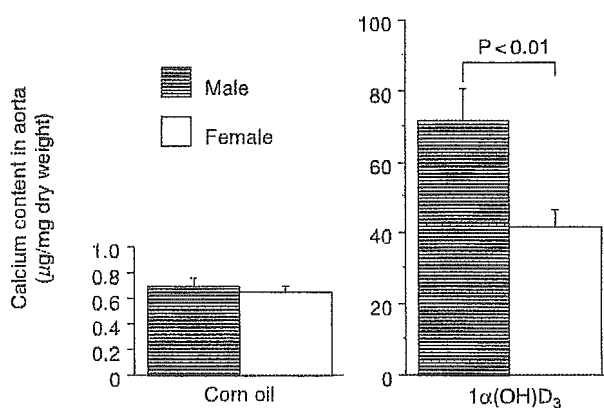


Figure 2 Sex difference in vitamin D toxicity-induced arterial calcification in rats. Vehicle or 5 $\mu\text{g}/\text{kg}$ per day $1\alpha(\text{OH})\text{D}_3$ was given to 30-week-old male and female rats for 2 weeks. Calcium content in the aorta was then measured and normalized by dry weight. $n = 8$ for corn oil and $n = 13$ for $1\alpha(\text{OH})\text{D}_3$ 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: bisphosphonates²¹ and osteoprotegerin⁵ 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 $1\alpha(\text{OH})\text{D}_3$ 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 $\mu\text{g}/\text{kg}$ per day $1\alpha(\text{OH})\text{D}_3$ 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 $\mu\text{g}/\text{kg}$ per day $1\alpha(\text{OH})\text{D}_3$ 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 $\mu\text{g}/\text{kg}$ per day $1\alpha(\text{OH})\text{D}_3$ 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 α (OH)D ₃	14.2 ± 0.3*	124 ± 33
OVX	Corn oil	10.7 ± 0.1	40 ± 6 [§]
	1 α (OH)D ₃	14.8 ± 0.3*	37 ± 7 [§]
OVX + E2	Corn oil	10.9 ± 0.2	95 ± 15
	1 α (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 α (OH)D₃ treatment.

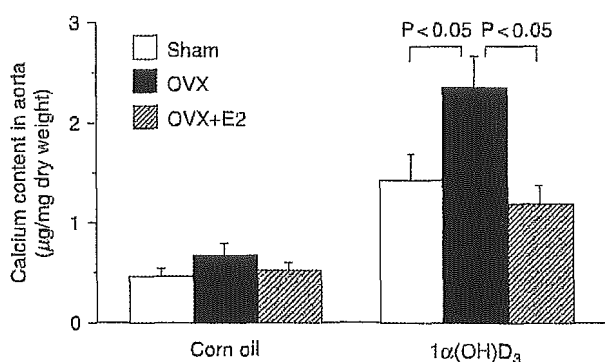


Figure 3 Effects of ovariectomy and estrogen replacement in vitamin D toxicity-induced arterial calcification in female rats. Vehicle or 5 μ g/kg per day 1 α (OH)D₃ was given to 8-week-old sham-operated (Sham), ovariectomized (OVX), and OVX + estradiol-replaced (OVX + E2) female rats for 2 weeks. Calcium content in the aorta was then measured and normalized by dry weight. n = 8 for corn oil and n = 12 for 1 α (OH)D₃ 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 μ g/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 resorption²⁴ and arterial calcification (our present results). In mice deficient in osteoprotegerin,²⁵ matrix Gla protein²⁶ 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 level²⁸ and was increased by estrogen replacement²⁹ 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.

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Original article

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

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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 $57.8 \pm 11.6 \mu\text{M}$. 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.

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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

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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 (\pm SD) aged 56 ± 4 (range 35–66)) and 24 age-matched controls (20 men; 4 women, mean (\pm 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 (angiotensin-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.

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 (Respirtrace 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

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 ($\text{wt}/(\text{ht})^2$)	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 \pm 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.

oxygen administration. Blood samples were collected in ice-cooled tubes containing lithium–heparin. Samples were deproteinized before analysis with 4% ZnSO₄. 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 NO₂⁻ plus NO₃⁻ concentrations, expressed in μM. In most samples, NO₃⁻ 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 SaO₂ in patients with OSAS was not different from that in the control subjects, the nadir SaO₂ 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 SaO₂ was markedly reduced (Table 2). The nadir SaO₂ 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 SaO₂ (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 SaO₂, systolic blood pressure, diastolic blood pressure, and arterial oxygen. There were significant relationships between NO_x levels, nadir SaO₂, 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 SaO₂, stable value of SaO₂ at supine position before sleep (%); nadir SaO₂, the lowest value of SaO₂ during night (%); N of 4% desaturation, number of 4% of arterial oxygen desaturation from baseline value of SaO₂.

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

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

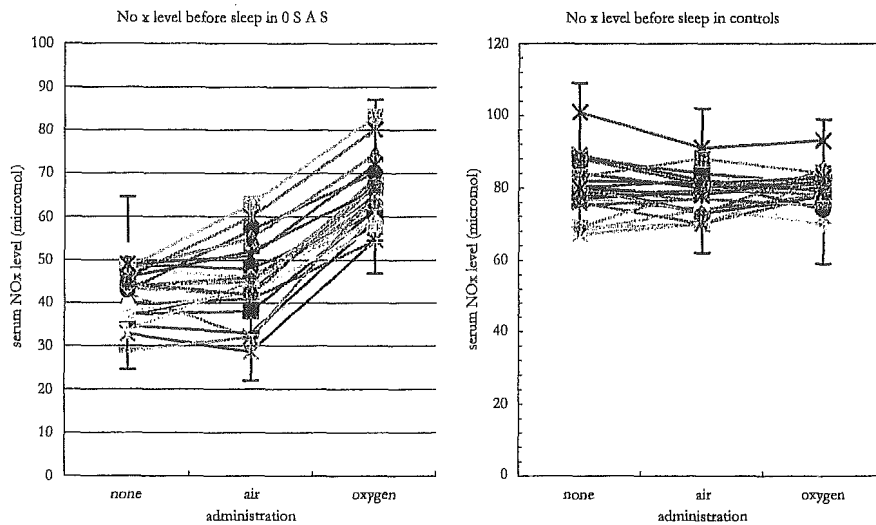


Fig. 1. Serum NO_x levels before sleep in patients with OSAS and in obese controls without OSAS.

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 SaO₂, 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

	<i>p</i>	<i>r</i>
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 O₂ 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.

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

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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 (\pm SD) aged 54.2 ± 3.6 (range 35–66)] and 24 age-

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

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matched controls [20 men : 4 women, mean (\pm 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 apneas/hypopneas without 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 VEGF₁₆₅, 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 \pm 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), FEV₁: forced expiratory volume in one second (l), PaO₂: arterial pressure of oxygen, PaCO₂: 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 SaO₂ in patients with OSAS was nearly identical to that in the control subjects, the nadir SaO₂ 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 SaO₂ in our patients was markedly reduced by oxygen administration (Table 2). Both nadir SaO₂ and mean SpO₂ 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 SaO₂ (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 SaO₂: stable value of SaO₂ at supine position before sleep (%), Nadir SaO₂: the lowest value of SaO₂ during night (%), N of 4% desaturation: number of 4% of arterial oxygen desaturation from baseline value of SaO₂, Mean SpO₂: the mean value of SaO₂ 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.

Table 4. Relationships between the Serum Levels of VEGF and Other Variables in All subjects

	P	r
AHI (/hour)	0.05	r= 0.401
Baseline SaO ₂ (%)	0.27	r=-0.223
Nadir SaO ₂ (%)	0.01	r=-0.457
N of 4% desaturation	0.01	r= 0.581
Mean SaO ₂ (%)	0.05	r=-0.389
Systolic BP	0.34	r= 0.027
Diastolic BP	0.41	r= 0.123

BP: blood pressure.

VEGF level (Table 3).

In examining the relationship between VEGF levels and numerous of parameters (AHI, nadir SaO₂, systolic blood pressure, diastolic blood pressure, arterial oxygen desaturation), we found that there were significant relationships between VEGF levels and AHI, nadir SaO₂, mean SpO₂, and the number of 4% oxygen desaturation (Table 4).

Discussion

The present study demonstrates that the administration of 2 l/min oxygen during the night to OSAS patients improves nocturnal hypoxemia in parallel with a decrease in VEGF levels. Serum VEGF levels were greater in obese patients with OSAS than in obese control subjects. Therefore, we conclude that circulating VEGF levels are elevated in OSAS patients, primarily due to nocturnal hypoxemia. Nevertheless, numerous factors might influence VEGF serum levels and must be taken into consideration when discussing these observations. The most common conditions known to be associated with elevated VEGF serum levels, such as disseminated cancer and chronic inflammatory and autoimmune disease, were not present in either the patients or the control subjects in the current study (17).

It has been reported that serum VEGF levels are significantly higher in patients with polysomnographically confirmed OSAS (AHI>15 and AI>5 in adults and children, respectively) than in patients with mild disease or no disease (18, 19). Furthermore, significant correlations have been found between VEGF concentrations and respiratory disturbance index and sleep time spent at SpO₂ <90% (18). Schulz et al reported that serum levels of VEGF are elevated in severely hypoxic patients with OSAS and are related to the degree of nocturnal oxyhemoglobin desaturation (20). They speculated that the most likely trigger of VEGF release in OSAS is hypoxia, since a close linear relationship between the degree of nocturnal oxyhemoglobin desaturation and VEGF concentrations was observed (20). If this is true, the administration of oxygen to patients with OSAS could reduce their VEGF levels to the normal levels found in subjects without OSAS. In the present study, we found that nighttime administration of oxygen, but not compressed air,

decreased the VEGF level and improved nocturnal oxyhemoglobin desaturation, but did not affect the occurrence of nocturnal apneas. It is reasonable to assume that repeated episodes of nocturnal hypoxemia are the primary mechanism of the increased production of VEGF in patients with OSAS.

However, previous studies have suggested that AHI is correlated with VEGF levels in OSAS patients (20). Because the administration of oxygen to OSAS patients improves both VEGF levels and nocturnal hypoxemia but not AHI, the relationship between AHI and VEGF levels may be associated with AHI-related nocturnal hypoxemia, but not directly with the number of apneas/hypopneas.

Other mediator systems related to hypoxia may also be involved in the mechanism of VEGF increase in patients with OSAS. Because reactive oxygen species and endothelin are elevated in OSAS patients (21, 22), these mediators may enhance gene expression of VEGF (23, 24). Because nitric oxide synthesis is downregulated in OSAS patients (25, 26), the inhibitory effect of nitric oxide on VEGF gene induction could be weakened in these patients.

The clinical significance of elevated VEGF concentrations in patients with sleep apnea remains a matter of speculation at this stage. VEGF is a mitogen specific for endothelial cells, which appears to play a pivotal role in physiological and pathological angiogenesis (13). VEGF has been shown to be an important factor in the pathogenesis of vascular-related diseases, including the growth of tumors, vascular dysfunction in diabetes mellitus, and atherosclerosis of the coronary arteries (13, 27, 28). The increased VEGF production found in OSAS patients might contribute to new vessel formation in ischemic and atherosclerotic vascular regions. Thus, enhanced VEGF production may constitute an adaptive mechanism to counterbalance the emergence of cardiovascular disease in patients with OSAS. The augmented VEGF concentration in sleep apnea patients having comorbidities may reflect a contributory mechanism for the development of cardiovascular disease. Recent data have suggested that amelioration of nocturnal hypoxia by nasal continuous positive airway pressure (nCPAP) is associated with a significant decrease in morning VEGF concentrations (29). The alterations of the VEGF system in patients with OSAS may have an impact on the development of cardiovascular abnormalities in these patients. Therefore, a reduction of VEGF concentration following treatment of sleep apnea and potentially reduced angiogenesis may be a clinically significant goal of treatment.

In conclusion, serum levels of VEGF are elevated in patients with OSAS and are closely correlated with the degree of nocturnal oxyhemoglobin desaturation. This increased VEGF was decreased by nighttime oxygen administration in OSAS patients, suggesting that nighttime hypoxia is a primary mechanism of VEGF increase in these patients. However, further studies are necessary to explore the clinical significance of VEGF increase on outcome in terms of cardiovascular diseases and mortality in OSAS patients.

Acknowledgements: This study was supported by research grants from Clinical Research for Evidenced-Based Medicine from Ministry of Health Labour and Welfare, Japan Arteriosclerosis Prevention Fund, and Foundation of Total Health Promotion.

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