

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, $8.2 \pm 1.0\%$; $n=6$, $p < 0.05$).

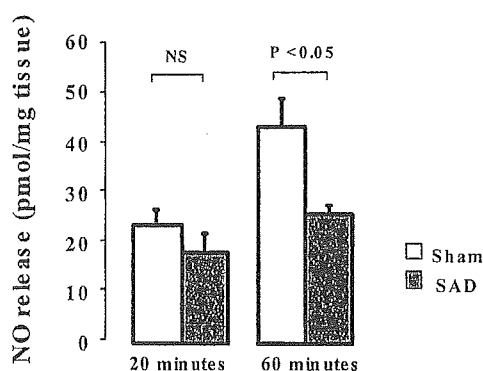


Fig. 5. Acetylcholine (1 μ mol/l)-induced NO production by isolated aortic rings derived from sinoaortic-denervated rats (SAD) and sham-operated rats (Sham). Each bar represents the mean \pm SEM ($n=5-7$ for each group).

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

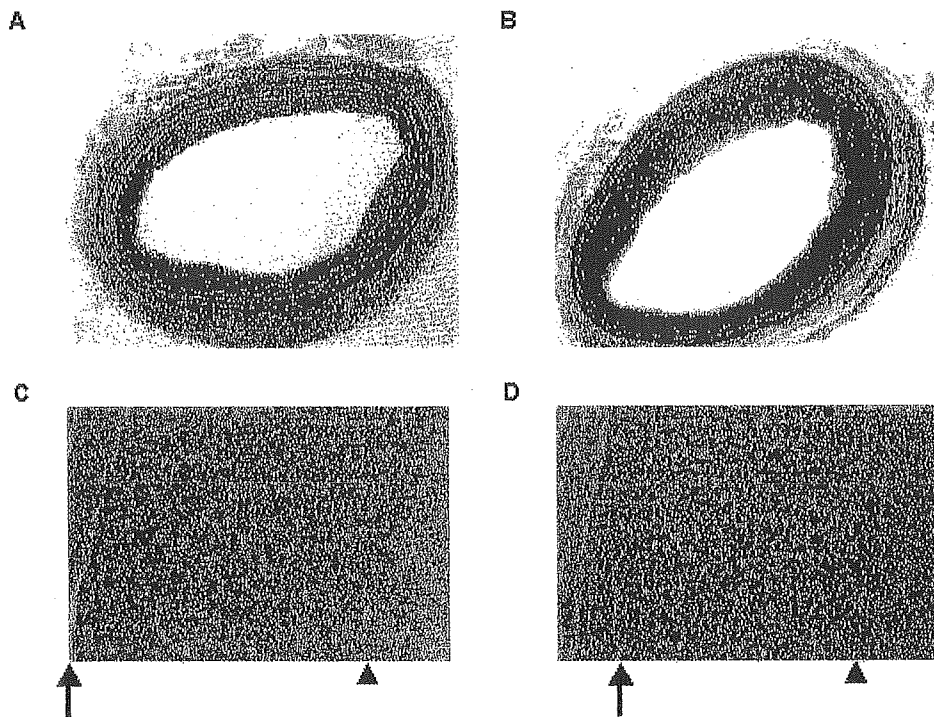


Fig. 6. Representative histological sections of common carotid arteries 14 days after balloon injury from a sham-operated rat (A, C) and a sinoaortic-denervated rat (SAD) (B, D). Elastica van Gieson staining (A, B) and immunohistochemical staining using anti-proliferating cell nuclear antigen (C, D).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

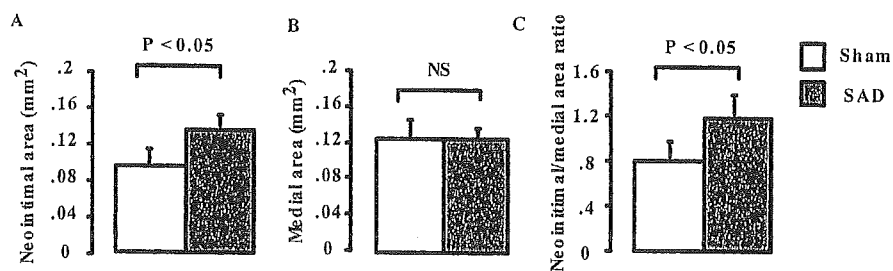


Fig. 7. A and B show cross-sectional areas of the neointima and media, respectively, of carotid arteries 14 days after balloon injury from sinoaortic-denervated rats (SAD) and sham-operated rats (Sham). C shows the neointimal-to-medial area ratio of carotid arteries from the same two groups. Each bar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

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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

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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).

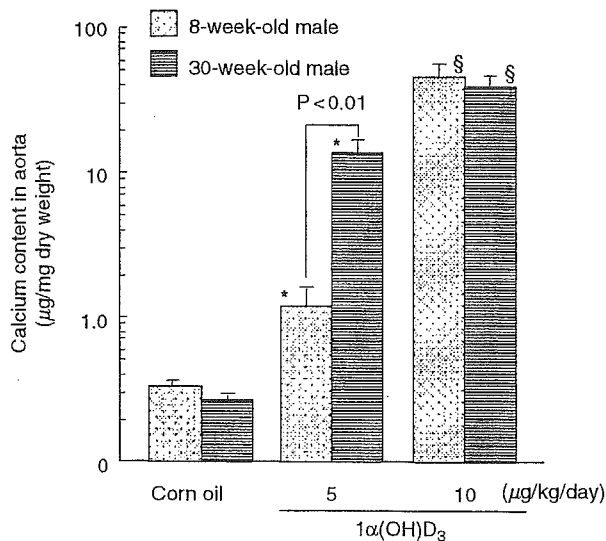


Figure 1 Influence of age and vitamin D dosage on arterial calcification in male rats. Vehicle or $1\alpha(\text{OH})\text{D}_3$ (5 or 10 $\mu\text{g}/\text{kg}$ per day) was given to 8-week-old and 30-week-old male rats for 2 weeks. Calcium content in the aorta was then measured and normalized by dry 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.

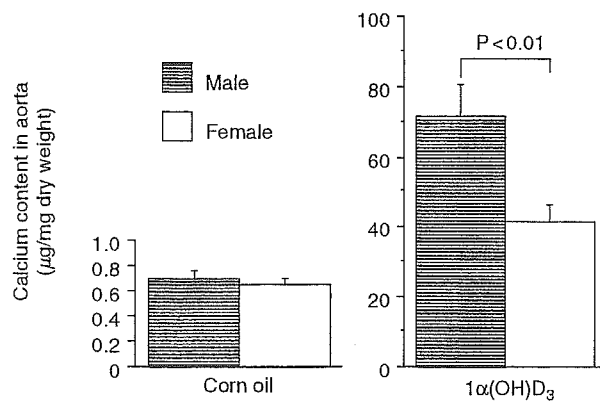


Figure 2 Sex difference in vitamin D toxicity-induced arterial calcification in rats. Vehicle or 5 $\mu\text{g}/\text{kg}$ per day $1\alpha(\text{OH})\text{D}_3$ was given to 30-week-old male and female rats for 2 weeks. Calcium content in the aorta was then measured and normalized by dry weight. $n = 8$ 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.

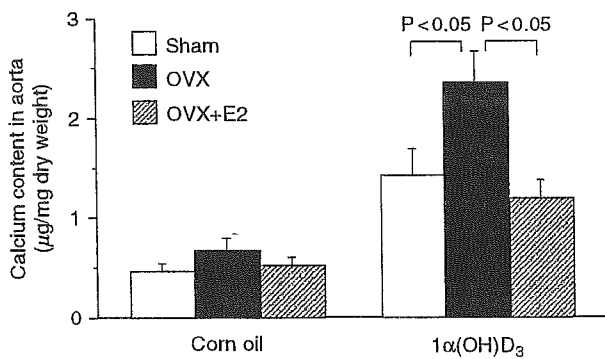


Figure 3 Effects of ovariectomy and estrogen replacement in vitamin D toxicity-induced arterial calcification in female rats. Vehicle or 5 μ g/kg per day 1 α (OH)D₃ was given to 8-week-old sham-operated (Sham), ovariectomized (OVX), and OVX + estradiol-replaced (OVX + E2) female rats for 2 weeks. Calcium 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

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17 β -Estradiol inhibits cardiac fibroblast growth through both subtypes of estrogen receptor

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Abstract

The effect of 17 β -estradiol (E2) on the proliferation of cardiac fibroblasts (CFs) remains controversial. This study investigated which subtype of estrogen receptor (ER), ER α or ER β , mediated the effect of E2 on CF growth by the gain of function analysis using an adenovirus vector. One hundred nanomoles per liter of E2 attenuated DNA synthesis by up to 10%, and transactivated the estrogen-responsive element determined by luciferase assay in rat neonatal CFs. We constructed replication-deficient adenoviruses bearing the coding region of human ER α , ER β , or the dominant-negative form of ER β (designated AxCAER α , AxCAER β , and AxCADNER β , respectively). When CFs were infected with AxCAER α or AxCAER β at multiplicity of infection of 20 or higher, DNA synthesis was decreased by 50% in response to E2 and the effect was abolished by co-infection with AxCADNER β . Similarly, transcriptional activity of ER in CFs infected with AxCAER α or AxCAER β was markedly enhanced and co-infection with AxCADNER β abolished the effects. These results suggest that E2 inhibits CF growth and that both ER subtypes mediate the effect comparably and redundantly.

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Structural remodeling of the ventricular wall takes place in several cardiac disorders including acute myocardial infarction, cardiomyopathy, and hypertensive heart disease. Histopathologically, it is characterized by a structural rearrangement of components of the normal chamber wall that involves cardiomyocyte hypertrophy, proliferation of cardiac fibroblast (CFs), fibrosis, and cell death [1]. In the adult heart, CFs substantially constitute the non-myocyte cells [2] and contribute to cardiac remodeling by undergoing proliferation, depositing extracellular matrix proteins which are mainly produced by CFs in the myocardium, and eventually replacing myocytes with fibrotic scar tissue. CFs also produce matrix metalloproteinases, growth factors, and cytokines, all of which are involved in the maintenance

of myocardial structure, and in diseased hearts play pivotal roles in remodeling [3]. Recent studies have shown that the interactions between CFs and cardiomyocytes are essential for the progression of cardiac remodeling [3]. Thus, it is clinically important to inhibit CF growth in the process of cardiac remodeling.

From several epidemiological studies, estrogen (E2) is thought to have a protective effect against left ventricular hypertrophy which is an important cardiovascular risk factor for morbidity and mortality [4–6]. Premenopausal women have a lower prevalence of left ventricular hypertrophy than their age-matched male counterparts [4]. Left ventricular mass is significantly greater in men than in women even after indexing for body surface area [5,6]. Experimental studies have shown cardioprotective roles of E2 [7–10], however, the direct effect of E2 on cardiac cell growth remains to be determined. Previous studies have demonstrated that the

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exogenous administration of E2 either decreased [7], increased [11], or had no effect on DNA synthesis in cultured CFs [12,13].

Most biological effects of E2 are mediated by the estrogen receptor (ER). ER has two subtypes, classical ER α and newly identified ER β [14]. It is reported that both ER subtypes are expressed in CFs [13,15]. However, little is known about the involvement of ER in CF growth, although many transcriptional factors including nuclear receptors regulate the functions of CFs in the process of cardiac remodeling [3]. There is only one report showing that the inhibitory effect of E2 on CF growth is independent of ER [16]. Adenovirus-mediated gene transfer is a useful tool to clarify the precise role of a specific gene. We constructed replication-deficient adenovirus vectors carrying ER α , ER β or dominant-negative form of ER β . In this study, to determine the effect of E2 on CF growth and which ER subtype plays a pivotal role in the cell growth, we evaluated DNA synthesis in CFs overexpressing each ER subtype using adenovirus vector. Here we show that E2 attenuated DNA synthesis by up to 10% in rat neonatal CFs and that adenovirus-mediated overexpression of either of the ER subtypes in CFs augmented growth inhibition in a ligand-dependent manner.

Methods

Cell culture. Rat CFs were harvested from the heart of Wistar neonatal rats at birth, as previously reported by Zang et al. [17]. Briefly, the hearts were removed from neonatal rats and minced with scissors until very small pieces were produced. The pellet of minced tissue was then resuspended in 1% collagenase and incubated at 37°C for 2 h. Next, the tissue was resuspended in 0.25% trypsin and incubated at 37°C for 2 h. The digested tissue was resuspended in Dulbecco's modified Eagle's medium (DMEM; Nikken Bio Medical Laboratory, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) (Intergen, Purchase, NY), 25 mM Hepes (pH 7.4), penicillin (100 U/ml), and streptomycin (100 µg/ml) at 37°C in a humidified atmosphere of 95% air and 5% CO₂. Twenty-four hours later, the medium was aspirated and the fresh medium was added. CFs at 6–9 passages were used in the experiments. At the time of experiments, we used dextran-coated charcoal-stripped FBS (DCC-FBS) and phenol-red-free M199 medium to avoid contamination with steroids or estrogen receptor agonists.

Construction of adenovirus vectors carrying estrogen receptor subtypes and transfer into CFs. Replication-deficient adenovirus vectors carrying the CMV-IE enhancer, chicken β -actin promoter, and the coding region of human ER α , ER β , or dominant-negative form of ER β , were constructed by use of adenovirus expression vector kit (Takara Shuzo, Kyoto, Japan) as described before [18] and named AxCAER α , AxCAER β , and AxCADNER β , respectively. CFs were exposed to different multiplicities of infection (MOI) of either AxCAER α , AxCAER β , AxCADNER β , or a replication-deficient recombinant adenovirus carrying the *Escherichia coli* β -galactosidase gene (AxCALacZ) for 2 h in DMEM with 5% FBS. Then, the cells were rinsed with phosphate-buffered saline once and used for the experiments.

RNA isolation, reverse transcription polymerase chain reaction. Total RNA was prepared from CFs and rat ovary as positive control, using Isogen (Wako Pure Chemical Industries, Osaka, Japan). Then, 1 µg total RNA was reverse transcribed into cDNA and one-twentieth of the product was amplified for 35 cycles. Negative control reverse

transcription polymerase chain reactions (RT-PCRs) were performed by omitting reverse transcriptase. The primer pairs used in PCR are: CTAAGAAGAATAGCCCCGCC (forward, +1126 to +1145) and CAGACCAGACCAATCATCAGG (reverse, +1402 to +1382) for rat ER α (GenBank Accession No. NM_012689), and CGACTGAGCAC AAGCCCAAATG (forward, +76 to +97) and ACGCCGTAA TGATACCCAGATG (reverse, +353 to +332) for rat ER β (GenBank Accession No. AB012721).

Measurement of [³H]thymidine incorporation. CFs seeded onto 24-well tissue culture plates were grown until 70–90% confluent and then made quiescent by culturing in phenol-red-free M199 medium (Gibco) for 24 h. Then, the cells were stimulated with 5% DCC-FBS in the presence of water-soluble 17 β -estradiol (Sigma–Aldrich, Japan) for 24 h, followed by pulse-labeling with 1 µCi/ml [³H]thymidine for 3 h. [³H]Thymidine incorporated into DNA was determined as previously described [19].

Number of CFs. CFs were seeded onto six-well multiplates and cultured until a confluent state was obtained. After infection of CFs with adenovirus vectors, the medium was replaced with phenol-red-free M199 to arrest the growth. After 24 h, the medium was replaced again with phenol-red-free M199 containing 5% DCC-FBS with E2 or vehicle. After incubation for 48 h, the cells were trypsinized and suspended. Then the number of cells was determined using a Coulter Counter (model ZM, Coulter Electronics, Hialeah, FL).

Luciferase assays. CFs were transfected with ERE-TK-Luc reporter plasmid and pRL-SV40 control plasmid using FuGENE6 (Roche) for 24 h according to the manufacturer's instructions [20]. Then, CFs were incubated in phenol-red-free M199 medium with 1% DCC-FBS for 24 h and exposed to E2 for additional 24 h. We measured two kinds of luciferase activity using a dual-luciferase reporter assay system (Promega) according to the manufacturer's protocol, and the ratio of firefly luciferase activity to that of *Renilla* luciferase in each sample was used as a measure of normalized luciferase activity [20].

Western blotting. After infection with adenovirus vector, cells were incubated with serum-free M199 medium for 24 h to detect ER subtypes. Cells were washed quickly with phosphate-buffered saline twice and lysed in RIPA buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitors cocktail; Complete, Mini; Roche). The samples were separated on 12% SDS-PAGE, electroblotted onto nitrocellulose membrane, and immunoblotted with anti-ER α polyclonal antibody (H-184; Santa Cruz, 1:1000 dilution), anti-ER β monoclonal antibody (CWK-F12, kindly provided by Dr. Benita S. Katzenellenbogen, thanks and details are given in Acknowledgements, 1:1000 dilution). Antibody was detected with a horseradish peroxidase-linked secondary antibody using an enhanced chemiluminescence system (Amersham Life Science).

Statistical analysis. The dose–response effect of E2 or ER overexpression on DNA synthesis in CFs was analyzed using one-way factor ANOVA. If a statistically significant effect was found, Newman–Keuls test was performed to isolate the difference between the groups. A value of $P < 0.05$ was considered statistically significant. All data in the text and figures are expressed as means \pm SE.

Results

Endogenous expression of ER subtypes and the effect of E2 on CF growth

To investigate the endogenous expression of ER in rat CFs, RT-PCR amplification was performed. Both rat ER α and ER β were expressed in CFs (Fig. 1A). At physiological concentrations, E2 inhibited the proliferation of CFs dose-dependently by up to 10% (Fig. 1B).

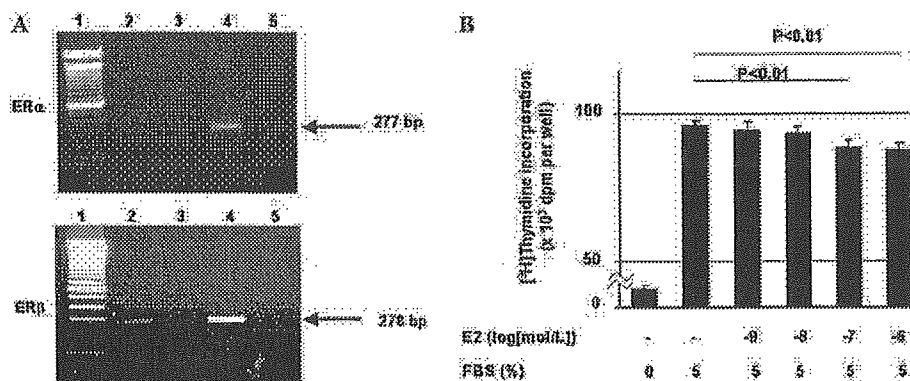


Fig. 1. ER gene expression and the effect of E2 on rat CF proliferation. (A) RT-PCR was performed using the cDNA of rat ovary as a positive control with (lane 4) or without (lane 5) reverse transcriptase and using the cDNA of rat CFs with (lane 2) or without (lane 3) reverse transcriptase. Lane 1 shows the molecular weight marker. (B) Serum-starved CFs were stimulated with 5% DCC-FBS in the absence or presence of 10–1000 nmol/L 17 β -estradiol for 24 h. [3 H]Thymidine incorporation into DNA was determined by pulse-labeling for the last 3 h of incubation. Results are shown as means \pm SE ($n = 3$). Similar results were obtained in three independent experiments.

Expression of ER subtype in CFs by adenovirus-mediated transfer of the ER subtype genes

Expression of the ER α and ER β protein was confirmed by Western blot analysis (Fig. 2A). Although both ER subtypes were detected by RT-PCR, the protein expression was undetectable in non-transfected CFs; the bands corresponding to ER α (65 kDa) or ER β (55 kDa) were seen in CFs infected with AxCAER α , or with AxCAER β , respectively (Fig. 2A), and also in MCF-7 cells or rat ovary which were used as positive controls (data not shown). The protein expression was increased by overexpression MOI-dependently. We also checked the protein level of both ER subtypes in non-infected cells after the addition of E2. However, E2 did not induce the protein of either ER subtype in our experimental conditions (data not shown).

Effect of adenovirus-mediated transfer of the ER subtype genes on CF growth

When AxCALacZ was introduced into CFs at more than 60 MOI, DNA synthesis reduced in a MOI-dependent manner in the absence of E2 (data not shown). Therefore, we examined DNA synthesis at 60 MOI or less to avoid the influence of adenovirus itself on DNA synthesis. CFs infected with AxCALacZ showed no additional decrease in DNA synthesis in response to E2 (Fig. 2B). In contrast, when CFs were infected with AxCAER α or AxCAER β at more than 10 MOI, DNA synthesis was significantly inhibited in a MOI-dependent manner in response to E2 to grossly similar extent. To confirm this, the cell number was counted in the presence or absence of E2. Comparable to the thymidine incorporation assay, overexpression of either ER α or ER β enhanced the inhibitory effect of E2 on CF growth (Fig. 2C). Moreover, in CFs infected with either AxCAER α or AxCAER β at 20 MOI, E2

decreased DNA synthesis in a concentration-dependent manner at 10 $^{-11}$ –10 $^{-6}$ mol/L (Fig. 3). Taking these results together, the effects of AxCAER α and AxCAER β seemed comparable. To examine whether the effect of ER transfer is truly ER subtype dependent, we investigated DNA synthesis in CFs co-infected with AxCAER α or AxCAER β and AxCAERDN β . The reduction of DNA synthesis in CFs infected with AxCAER α or AxCAER β alone at 20 MOI was abolished by co-infection with AxCAERDN β (Fig. 4).

Transcriptional activity of ERE in CFs infected with ER genes

We examined the transcriptional activity of ER by luciferase activity of the ERE reporter plasmid. In non-infected CFs, 100 nmol/L E2 augmented the luciferase activity of ERE by approximately 1.3-fold compared to vehicle ($p = 0.02$) (Fig. 5). CFs infected with AxCAER α or AxCAER β at 20 MOI showed a strong increase in transcriptional activity in the presence of E2; 3.3-fold increase with AxCAER α and 3.9-fold increase with AxCAER β in response to E2. This increase was completely abolished by co-infection with AxCADNER β .

Discussion

Conflicting results have been reported concerning the effect of E2 on CF growth. One group demonstrated that CF growth was not affected by E2 [12]. Two groups showed that E2 inhibited CF growth [7,13], whereas another has shown that E2 enhanced CF growth through mitogen-activated protein kinase-dependent pathway [11]. Thus, the effect of estrogen on CF growth remained to be addressed. In this study, E2 inhibited DNA synthesis in CFs by up to 10%, and this inhibition was augmented by overexpression of either of ER subtypes,

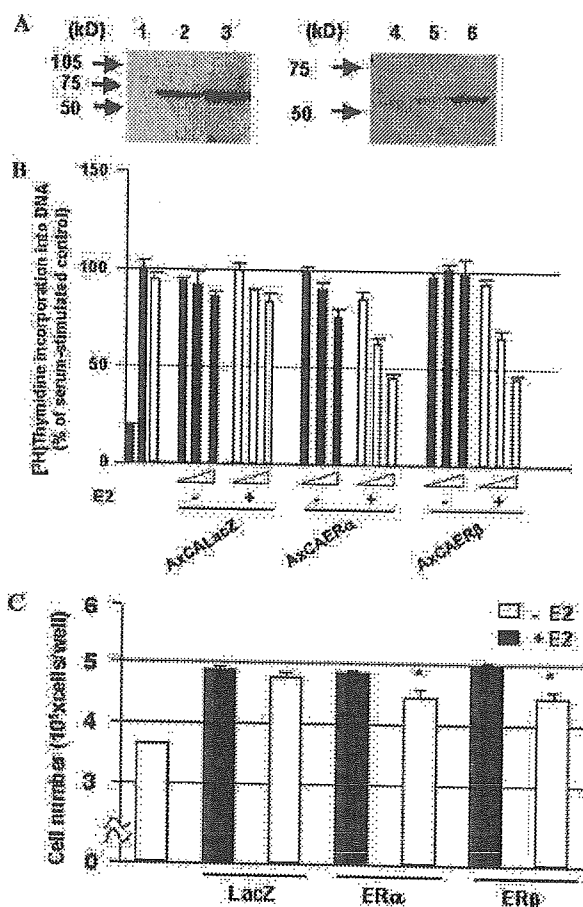


Fig. 2. Induction of ER protein and inhibition of CF growth by adenovirus-mediated transfer of ER genes. (A) CFs were infected without (lanes 1 and 4), or with 10 and 100 MOI of AxCAER α (lanes 2 and 3, respectively) or 10 and 100 MOI of AxCAER β (lanes 5 and 6, respectively). Western blot analysis was performed with 40 μ g of protein per lane by using an anti-ER α polyclonal antibody (left panel) or anti-ER β monoclonal antibody (right panel). CFs seeded onto a 24-well plate (B) or 6-well plate (C) were exposed to DMEM containing either AxCALacZ, AxCAER α , or AxCAER β (1, 10, and 30 MOI (B), respectively, from left to right, or 30 MOI (C)) for 2 h and serum-deprived for 24 h. [³H]Thymidine incorporation into DNA (B) was determined at 24 h after the stimulation with 5% DCC-FBS in the presence or absence of 100 nmol/L E2 and presented as a percentage of the serum-stimulated control. The left-sided 3 lines indicate non-infected CFs with serum-free medium, 5% DCC-FBS in the absence of E2, and 5% DCC-FBS in the presence of 100 nmol/L E2, respectively (B). Cell numbers were counted after 48 h of stimulation with 5% DCC-FBS in the presence or absence of 100 nmol/L E2 (C). The left-sided line indicates non-infected CFs before the stimulation. * $P < 0.01$ vs CFs without E2. Results are shown as means \pm SE ($n = 3$) (B,C). Similar results were obtained in three independent experiments.

indicating that both ER subtypes work to inhibit CF growth in a redundant fashion.

Both ER subtypes are expressed in cardiac myocytes and CF as shown by Western blotting [13,15,21], and are transcriptionally active [15], suggesting that ER subtypes play a role in cardiac cells. Moreover, it is reported that the expression of ER subtypes in cardiac cells was regulated by physiological or pathophysiological stimuli

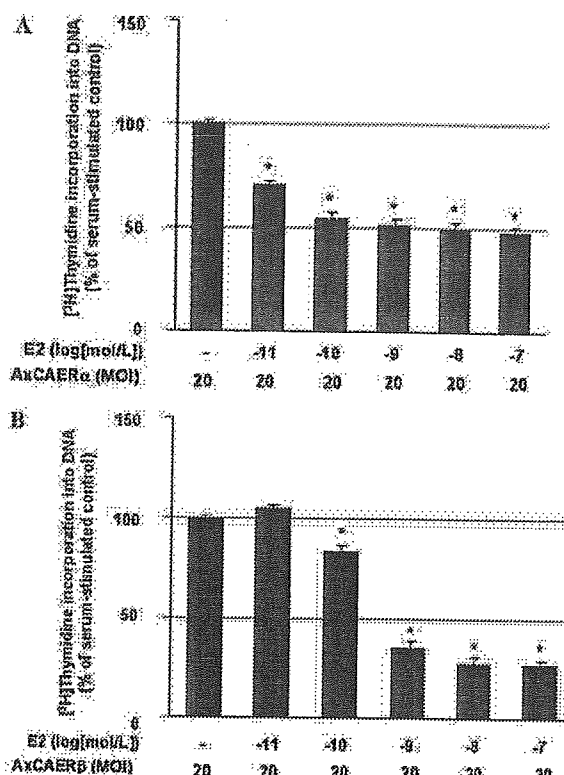


Fig. 3. Dose-response effect of E2 on DNA synthesis in CFs overexpressing ER subtypes. CFs seeded onto a 24-well plate were exposed to DMEM containing 10 MOI of AxCAER α (A) or AxCAER β (B) for 2 h, and were serum-deprived for 24 h. [³H]Thymidine incorporation into DNA was determined at 24 h after the stimulation with 5% DCC-FBS in the absence or presence of the indicated concentrations of E2 and presented as a percentage of CFs without E2. Results are shown as means \pm SE ($n = 4$). Similar results were obtained in three independent experiments.

such as E2 [15] and hypoxia [22]. Protein levels of both ER subtypes were increased in CFs and cardiac myocytes in response to E2 [15]. Under hypoxic condition, the protein level of ER β but not of ER α was upregulated while the presence of E2 decreased the level of ER β protein in CFs [22]. Modulation of ER subtype expression by E2 was not confirmed in the present study (data not shown) presumably because the expression in non-transfected CFs was too low to detect by Western blotting. Changes of ER expression in cardiac cells associated with cardiovascular disease are currently unknown. However, the gain-of-function analysis implies the physiological relevance by mimicking the conditions of the previous reports [13,15,21,22]. Another rationale in using the overexpression system was to compare the effects on CF growth between ER subtypes. Because adenovirus vectors successfully induced ER subtypes to a similar extent, we could interpret the results clearly.

Several reports have examined the role of ER subtypes in proliferation using the gene transfer techniques into cell lines [23–26]. Cheng and Malayer [26] have reported that overexpression of ER α in an ER-negative

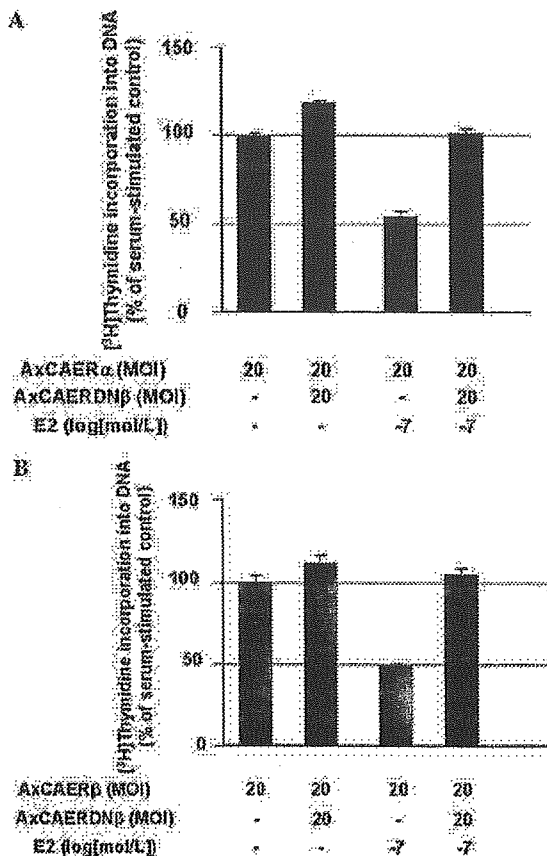


Fig. 4. The effect of dominant negative ER on ER subtype overexpression in CFs. CFs seeded onto a 24-well plate were exposed to DMEM containing 20 MOI of AxCαERα (A) or AxCαERβ (B) and the indicated MOI of AxCADNERβ. After infection, CFs were serum-deprived for 24 h. [³H]Thymidine incorporation into DNA was determined at 24 h after the stimulation with 5% DCC-FBS in the absence or presence of 100 nmol/L of E2, and were presented as a percentage of CFs infected with AxCαERα alone (A) or AxCαERβ alone (B) without E2. Results are shown as means ± SE (*n* = 3). Similar results were obtained in three independent experiments.

rat fibroblast cell line, rat-1, resulted in an estrogen-dependent small (<10%) but significant increase in cell proliferation but overexpression of ERβ did not affect proliferation. In contrast, Lazennec et al. [24] have shown that overexpression of ERα in an ER-negative human breast cancer cell line, MDA-MB-231, led to a hormone-dependent inhibition of proliferation, whereas overexpression of ERβ caused a hormone-independent inhibition. Taken these results together with other reports examining the effect of ER overexpression in non-CF cells [23–25], the role of ER subtypes in cell proliferation may be different between cell types. This may also be the case with our results and the results in fibroblasts by Cheng and Malayer [26]. We used neonatal primary cultured CFs that expressed low levels of both ER subtypes, while Cheng et al. used a cell line derived from embryo fibroblasts that did not express ER.

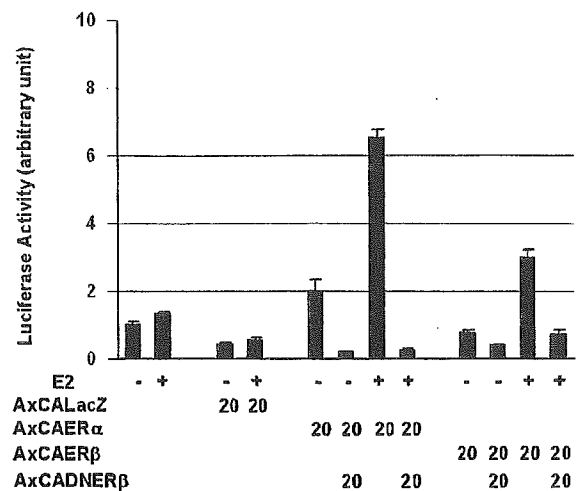


Fig. 5. The influence of ER overexpression on the promoter activity of ER responsive enhancer elements in CFs. CFs were infected with AxCALacZ, AxCαERα, AxCαERβ, or AxCADNERβ at the indicated MOI for 2 h, and transfected with the luciferase reporter plasmids containing ERE and the pRL-SV40 control plasmid. Twenty-four hours after transfection, the cells were treated with or without 100 nmol/L E2 for 24 h. Results are shown as means ± SE (*n* = 3). Similar results were obtained in three independent experiments.

The divergent roles of ER subtypes can be explained by the differential induction of estrogen response genes, the different interactions with promoter elements including AP-1 sites [27] and SP-1 sites [28] in an ERE-independent manner, or differential recruitment of transcriptional co-factors. In the present study, however, ERα and ERβ inhibited CF growth and transactivated the ERE similarly in response to E2. The only difference between ER subtypes observed in this study was that overexpression of ERα exerted the effects on cell growth and transcriptional activity ligand-independently (Figs. 2B and 5), although these effects were slight and might be non-specific. Accordingly, it is suggested that ERα and ERβ mediate the inhibitory effect of E2 on CF growth in a redundant or compensatory fashion as is the case with some gene superfamilies [29,30].

Our findings provide a mechanistic insight into the understanding of how E2 acts in CFs in the process of cardiac remodeling. Our data imply that the proliferation of CFs involved in cardiac hypertrophy and fibrosis can be inhibited by E2 as is shown in clinical and experimental settings [4–10], and that both ER subtypes expressed in CFs mediate the inhibitory effects of E2. Unfortunately, recent clinical trials [31,32] have failed to show beneficial effects of hormone replacement therapy on cardiovascular disease. Alternatively, specific ligands such as selective ER modulators [33] might exert beneficial clinical effects, particularly in combination with the gene transfer of ER subtypes, to inhibit cardiac remodeling. To test this possibility, in vivo experiments using ER overexpression and selective ER modulators should be performed in the future.

Acknowledgments

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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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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.

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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.

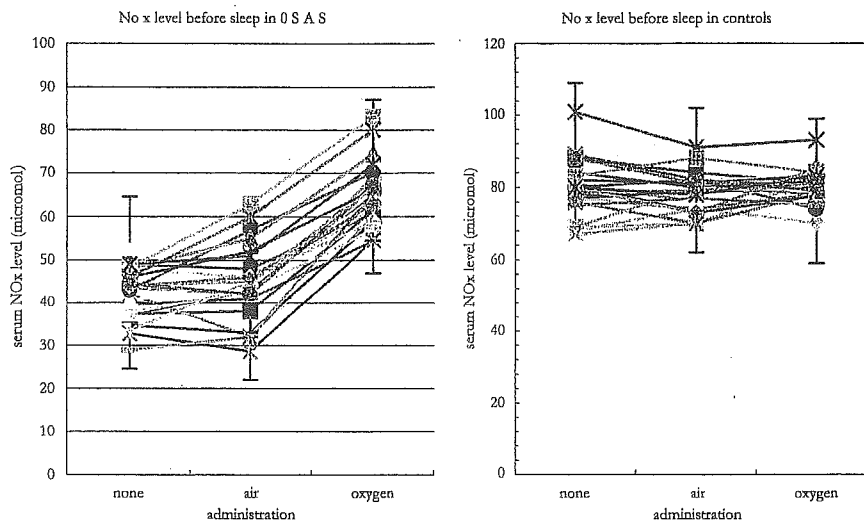


Fig. 1. Serum NO_x levels before sleep in patients with OSAS and in obese controls without OSAS.

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.

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