examined 18 h after the addition of 100 nmol/l E2. The expression was increased by the addition of serum and was attenuated in VSMCs infected with AxCAERβ in the presence of E2. In contrast, E2 did not significantly inhibit cyclin A protein expression in VSMCs infected with AxCALacZ or AxCAERα (Fig. 6).

4. Discussion

Our previous work and several articles by other investigators have clearly demonstrated that E2 inhibits the proliferation of VSMCs [5–8]. Recently, it was reported that ER antagonists, ICI182,780 [8] and tamoxifen [6], antagonized the inhibitory effect of estrogen, indicating that the effect was mediated by ER. However, these inhibitors are non-selective for the ER subtype, and tamoxifen exerts a partial agonistic effect in some tissues [29]. Thus, it remained unclear which receptor is involved in the inhibitory effect of estrogen on VSMC proliferation.

There are several reports focusing on the effect of estrogen on cell proliferation using adenoviruses carrying ER into the breast cancer cell line or the pituitary lactrope cell line [30–32]. In MDA-MB 231 cells, an ER-negative human breast cancer cell line, overexpression of ERα inhibited proliferation hormone-dependently, whereas that of ERβ inhibited proliferation ligand-independently [31]. Also, overexpression of wild-type ERα in a pituitary lactrope cell line inhibited proliferation and induced apoptosis [32]. In contrast to these reports, overexpression of the dominant-negative form of ERα inhibited the proliferation of MCF-7 cells, in which endogenous ERα was expressed, and the proliferation was increased in an estrogen-dependent manner [30]. Although the question of ERα overexpression resulting in growth inhibition depending on the cell line is unresolved, the use of recombinant adenoviruses in this study enabled us to induce ERα and ERβ abundantly in VSMCs in which the endogenous expression of both ER subtypes was low.

The present study demonstrates direct evidence that ERβ is involved in the control of VSMC proliferation. The inhibitory effect of ERβ overexpression was restored by co-infection of dominant negative ERβ, indicating that this phenomenon actually resulted from signaling via ERβ. This dominant negative form of ERβ has an inhibitory effect on the transcriptional activity of both wild-type ERα and ERβ, as demonstrated by Ogawa et al., who originally made these constructs [25]. They made the C-terminal truncated ERβ, and showed that this dominant negative form of ERβ can bind to both wild-type ERα and ERβ. Accordingly, the dominant negative ERβ we used can inhibit not only the homodimerization of wild-type ER, but also heterodimerization of ERα and ERβ. The downstream effect was unknown, although competition for ERE binding, formation of inactive heterodimers with wild-type ER and specific transcriptional silencing could be assumed.

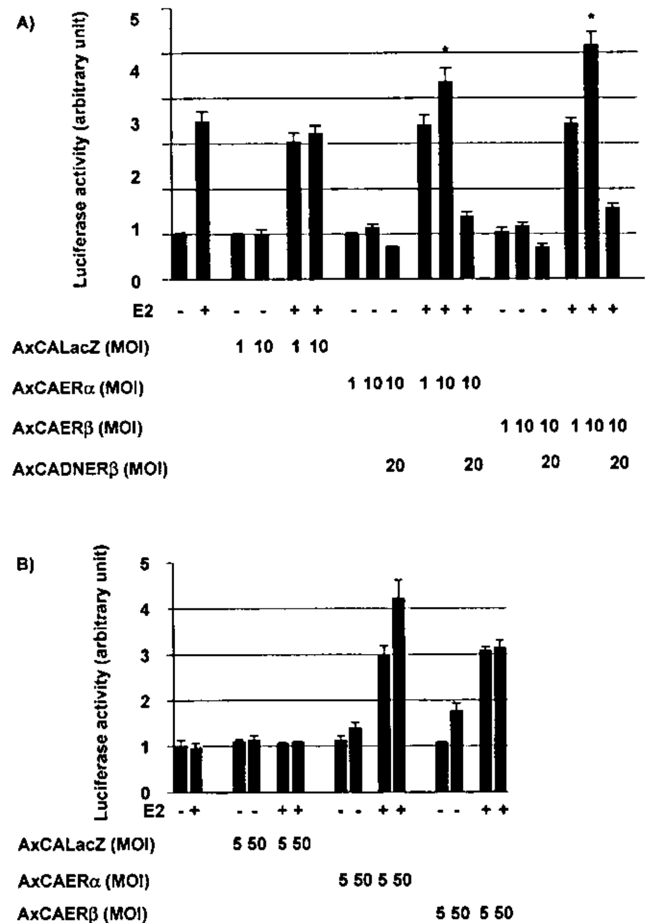

Fig. 5. Influence of overexpression of ERs on promoter activity of ER responsive enhancer elements in VSMCs and COS-7 cells. (A) VSMCs were infected with AxCALacZ, AxCAERα, AxCAERβ, or AxCADNERβ for 2 h, and were then transfected with luciferase reporter plasmids. Twenty-four hours after transfection, the cells were treated with or without 100 nmol/l E2 for 24 h. *$P<0.01$ vs. VSMCs infected with 10 MOI of AxCALacZ with E2. (B) COS-7 cells were infected with AxCALacZ, AxCAERα, or AxCAERβ for 2 h, and were then transfected with luciferase reporter plasmids. Twenty-four hours after transfection, the cells were treated with or without 100 nmol/l E2 for 24 h. The values were normalized to those in non-infected VSMCs without E2. Results are shown as mean±S.E. ($n=3$).

Surprisingly, the inhibitory effect of E2 was seen even at 10 pmol/l in VSMCs infected with ERβ. On the other hand, what is the role of ERα in VSMC proliferation? When ERα was infected into growth-arrested VSMCs, no proliferative response to E2 was seen (data not shown). Also, when both ERα and ERβ were co-infected into VSMCs, the inhibitory effect of ERβ was not affected. These results indicate that ERα does not show stimulatory effects or antagonize ERβ in terms of VSMC growth. When VSMCs were infected with AxCAERα at a higher MOI (30 or 100 MOI), an inhibitory effect appeared (Fig. 2A and data not shown). Taken together, ERα may have a weak inhibitory effect on VSMC proliferation.

The divergent effects of the ER subtypes may be

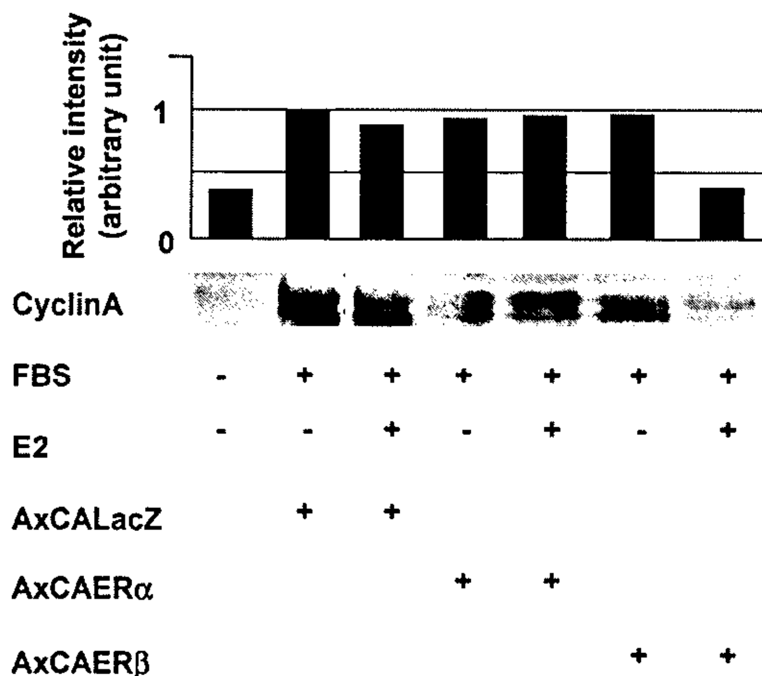

Fig. 6. Downregulation of cyclin A protein by E2 in VSMCs infected with AxCAERβ. VSMCs seeded onto 10 cm dishes were exposed to DMEM containing 10 MOI of AxCALacZ, AxCAERα, or AxCAERβ for 2 h. After infection, VSMCs were serum-deprived for 24 h. Samples were harvested 18 h after stimulation with 5% DCC-FBS in the absence or presence of 100 nmol/l E2. Western blot analysis was performed with 30 μg of protein per lane by using an anti-cyclin A polyclonal antibody. In the upper panel, levels of cyclin A protein expression in the membrane were measured by densitometry, and were plotted in comparison with that in serum-stimulated VSMCs without E2. Similar results were obtained in three independent experiments.

explained by the differential induction of estrogen response genes [33], or they may be due, in part, to the differential recruitment of transcriptional co-factors. A difference in ligand-binding affinity has also been reported [34]. Recently, it was also reported that ERβ, but not ERα, binds MAD2, a cell cycle spindle assembly checkpoint protein. The interaction is not altered by the absence or presence of E2, and thus ERβ is thought to function as a component of the spindle checkpoint assembly, not as a transcriptional factor [35]. With respect to cell growth, c-myc proto-oncogene expression was decreased in breast cancer cells infected with ERα, but was not changed in cells infected with ERβ, although the transcriptional activity is similar in cells infected with different ER subtypes [31]. In our study, there is also a discrepancy between the ERE luciferase activity and thymidine incorporation in terms of dose-dependency and the differential roles of ER subtypes. The reason for this is unknown, but similar findings have been reported [31]. We can put forward an hypothesis: some machinery, such as the co-factor for ER, would be limited in VSMCs, and the overexpression of ER could increase the ERE transcription activity to a small extent. By contrast, the signaling pathway mediating growth inhibition might manipulate some response gene that did not contain the typical ERE or non-genomic factors [6,36]. Thus we checked a cell cycle regulated gene, cyclin A. This molecule is important in the G1/S transition and in the S and G2/M phases of the cell cycle and plays a critical role in DNA replication [37]. Although the direct interaction between ERβ and cyclin A could not be clarified, cyclin A might be one of the specific response genes for ERβ in VSMCs. With respect to signaling pathways, it was reported that E2 had an inhibitory effect on VSMC proliferation via the inhibition of mitogen-activated protein kinase (MAPK) activity [6], an increase in the expression of MAPK phosphatase-1 and the activity of two Src homology 2 domain-containing cytosolic tyrosine phosphatases [38], or the cyclic AMP–adenosine pathway [7]. These signaling pathways are attributable, in part, to the non-genomic action of E2 [6,38]. We have tested whether the inhibition of MAPK activity could be involved in the inhibition of VSMC growth. However, under our study conditions, E2 did not affect ERK activity regardless of infection. Further investigations are required to clarify the specific signaling pathway by which ER subtypes exert differential effects on VSMC proliferation.

The in vivo relevance of our findings should be discussed. Studies on vascular injury using genetically engineered mice, such as ERα knockout [19], ERβ knockout [20], ERα and β double knockout mice [21] and fully null ERα knockout [22], are not yet conclusive in addressing the role of ER subtypes. In rats, ERβ is predominantly expressed in the aorta [39] or after injury to the carotid artery [23], and might play a more important role. To understand the more exact mechanism of action of ER in the vascular wall, we should make an effort to resolve this

discrepancy and are thus preparing a rat study to examine the effect of ERβ on VSMC proliferation in vivo.

Acknowledgements

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

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

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

Introduction

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

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

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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 $1\alpha(OH)D_3$ (dissolved in 0.5 mL corn oil; supplied by Teijin, Tokyo, Japan) using a feeding tube daily for 2 weeks. The other group of rats was orally given corn oil as vehicle for 2 weeks.

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

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

Assay for arterial calcification and serum calcium

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

Data analysis

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

Results

Effect of age and dosage of vitamin D

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

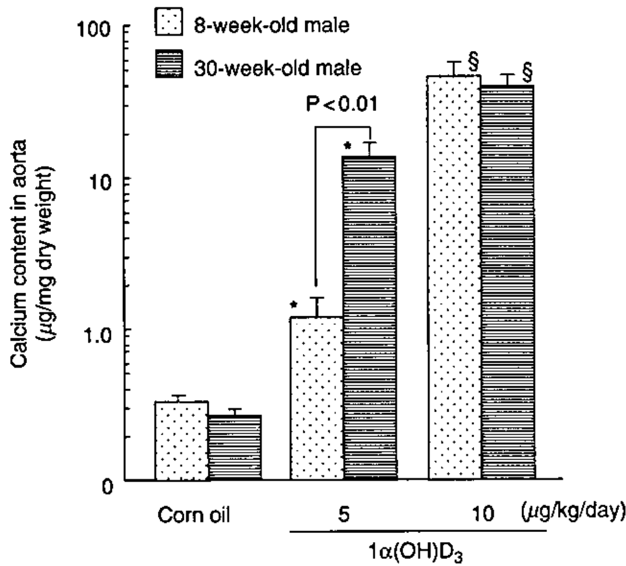

Figure 1 Influence of age and vitamin D dosage on arterial calcification in male rats. Vehicle or $1\alpha(OH)D_3$ (5 or 10 μg/kg/per ay) was given to 8-week-old and 30-week-old male rats for 2 weeks. Calcium content in the aorta was then measured and normalized by dry weight. *$P < 0.01$ *vs* corn oil,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

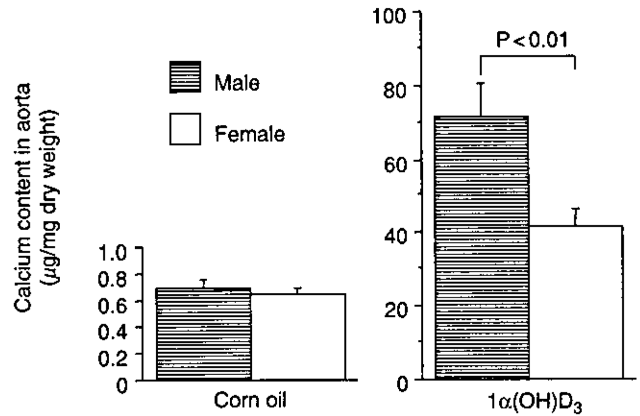

Figure 2 Sex difference in vitamin D toxicity-induced arterial calcification in rats. Vehicle or 5 μg/kg per day $1\alpha(OH)D_3$ was given to 30-week-old male and female rats for 2 weeks. Calcium content in the aorta was then measured and normalized by dry weight. $n = 8$ for corn oil and $n = 13$ for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

Sex difference and effects of estrogen

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

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

Discussion

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

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

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

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

	Treatment group	Serum calcium (mg/dL)	Estradiol (pmol/L)
Sham	Corn oil	10.8 ± 0.2	129 ± 30
	1α(OH)D$_3$	14.2 ± 0.3*	124 ± 33
OVX	Corn oil	10.7 ± 0.1	40 ± 6§
	1α(OH)D$_3$	14.8 ± 0.3*	37 ± 7§
OVX + E2	Corn oil	10.9 ± 0.2	95 ± 15
	1α(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 1α(OH)D$_3$ treatment.

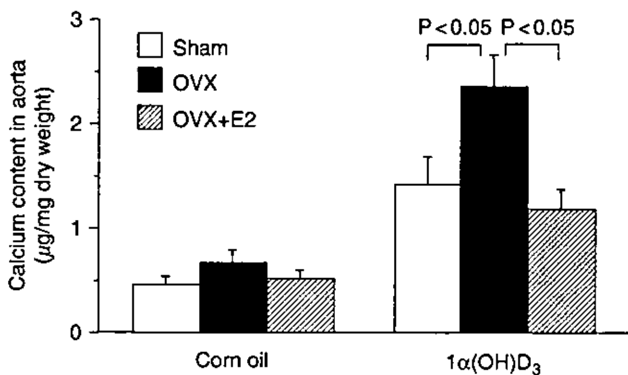

Figure 3 Effects of ovariectomy and estrogen replacement in vitamin D toxicity-induced arterial calcification in female rats.Vehicle or 5 μg/kg per day 1α(OH)D$_3$ was given to 8-week-old sham-operated (Sham), ovariectomized (OVX), and OVX + estradiol-replaced (OVX + E2) female rats for 2 weeks. Calcium content in the aorta was then measured and normalized by dry weight. n = 8 for corn oil and n = 12 for 1α(OH)D$_3$ treatment.

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

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

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

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

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

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LETTERS TO THE EDITOR

TESTOSTERONE AND COMPREHENSIVE GERIATRIC ASSESSMENT IN FRAIL ELDERLY MEN

To The Editor: A low plasma testosterone level in elderly men has been implicated in various diseases, including atherosclerosis, osteoporosis, and dementia,[1] but the relationship between plasma testosterone and functional measures in frail elderly men has not been addressed. Here, we report a small-scale study in which a low plasma testosterone

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level was associated with a functional decline based on comprehensive geriatric assessment in frail elderly men without malnutrition.

Fifty-four consecutive men (aged 70–95, mean age ± standard deviation = 82 ± 6 years) attending the health service facilities for the elderly (Mahoroba-no-Sato) located in Nagano Prefecture for admission or day-care service were enrolled. Of the subjects, five were diagnosed as having dementia of the Alzheimer type, six had history of stroke, five had a history of heart failure, eight had hypertension, and five had hyperlipidemia. Subjects with malnutrition, malignancy, or endocrine disease were excluded because these diseases may affect plasma androgen level. A commercial laboratory determined plasma total testosterone (total-T) and free testosterone (free-T) in addition to blood cell counts and blood chemical parameters. Total-T and free-T were assayed using a sensitive radioimmunoassay, and the intra-assay coefficients of variation were 5% to 6% and 2% to 5%, respectively. Basic activities of daily living (ADLs) were assessed using Barthel Index, instrumental activities of daily living (IADLs) by Lawton and Brody, cognitive function using Hasegawa Dementia Scale—Revised (HDS-R), mood using the Geriatric Depression Scale (GDS; 15 items), and ADL-related vitality using Vitality Index.[2]

On average, the subjects showed mild to moderate functional decline (Table 1). Also, mean plasma levels of total-T (365 ± 172 ng/dL) and free-T (5.7 ± 2.8 pg/mL) were lower than those reported in healthy elderly men[3] but comparable with those in frail elderly men.[4] As shown in Table 1, total-T and free-T were significantly correlated with functional measures except for GDS. There was no significant correlation between total-T or free-T and age, body mass index, blood hemoglobin, lymphocyte count, serum albumin, or serum total cholesterol, probably because the subjects had good nutritional status; serum albumin was 4.1 ± 0.3 g/dL, and serum total cholesterol was 179 ± 28 mg/dL. Furthermore, multivariate analysis with age, serum albumin, and serum total cholesterol as independent variables revealed that free-T was an independent determinant for the HDS-R ($R = 0.403$, $P = .03$) and Vitality Index ($R = 0.407$, $P = .02$). In similar multivariate analyses, free-T was not an independent determinant for the Barthel Index or IADLs, and total-T was not an independent determinant for each of the functional measures (data not shown).

The present study demonstrated that a higher plasma testosterone level was associated with higher scores of comprehensive geriatric assessment except for the GDS. Free-T, the active form of testosterone, showed a stronger correlation than did total-T. It has been reported that a higher total-T was associated with better ADL performance such as transferring and eating in frail elderly men.[4] The result is consistent with ours, but free-T and nutritional assessment were not included in that report. It is known that malnutrition is associated with low ADL and low plasma testosterone. In our preliminary study in elderly male patients in sanatorium-type wards, serum albumin was correlated with the Barthel Index and plasma total-T and free-T (data not shown). Consequently, nutritional markers such as serum albumin should be included as confounding factors in a study that examines the relationship between plasma testosterone and ADLs in frail elderly men. In fact, no significant relationship was found between plasma testosterone (total-T and free-T) and ADLs (Barthel Index and IADLs) in multivariate analyses including nutritional markers, although the subjects were well nourished. Alternatively, the correlation of free-T with cognitive function and ADL-related vitality remained significant in multivariate analyses. Contrary to a previous report,[5] depressed mood did not relate to plasma testosterone in this study. The reason is unknown, but it might be due simply to the cohort difference between community-dwelling healthy men[5] and frail elderly men or to the low reliability of GDS in demented people.[2]

The detailed and causal relationships need to be examined in large-scale and longitudinal studies. Nevertheless, our results suggest that testosterone treatment might improve global function in frail elderly men with low testosterone levels.

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Table 1. Distribution of Functional Measures and Correlation Coefficients Between Plasma Testosterone Level and Functional Measures

Measure	Barthel Index	IADL	HDS-R	GDS	Vitality Index
Mean ± standard deviation (range)	73 ± 27 (5–100)	2.1 ± 2.0 (0–5)	18 ± 7 (2–29)	6.3 ± 3.1 (1–13)	8.8 ± 1.8 (3–10)
Total testosterone	.422†	.279*	.344*	.077	.370†
Free testosterone	.369†	.390†	.512‡	.164	.464‡

Note: The Barthel Index was used to assess Activities of daily living, Lawton and Brody's instrumental activities of daily living (IADL) index to assess IADLs, the Hasegawa Dementia Scale—Revised (HDS-R) to assess cognitive function, the Geriatric Depression Scale (GDS—15 items) to assess mood, and the Vitality Index to assess ADL-related vitality.
* $P < .05$;
† $P < .01$;
‡ $P < .001$.

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Caveolin-1, Id3a and two LIM protein genes are upregulated by estrogen in vascular smooth muscle cells

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Abstract

Estrogen has diverse effects on the vasculature, such as vasodilation, endothelial growth and inhibition of vascular smooth muscle cell (VSMC) proliferation and migration. However, little is known about the genes that are regulated by estrogen in the vascular wall. Wistar rats were ovariectomized or sham-operated (Sham group), and 2 weeks after the operation, were subjected to subcutaneous implantation of placebo pellets (OVX + V group) or estradiol pellets (OVX + E group). Endothelium-denuded aortic tissue was examined 2 weeks after implantation. By applying high-density oligonucleotide microarray analysis, the expression of approximately 7000 genes was analyzed. Among the genes with different expression levels between the OVX + E group and the OVX + V group, those that have been reported to be expressed in the vasculature or muscle tissue, were chosen. Finally, four genes, caveolin-1, two LIM proteins (enigma and SmLIM) and Id3a, were identified. Microarray as well as real-time polymerase chain reaction showed that the expression levels of these genes were significantly higher in the OVX + E group than in the OVX + V group. To clarify whether estrogen directly upregulates these genes in the vascular wall, Northern blot analysis was performed using cultured rat VSMC. Addition of 100 nmol/L estradiol for 24 hours increased the mRNA levels of all four genes. Although the

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precise mechanism remains unclear, regulation of these genes by estrogen might contribute to its effect on VSMC.
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Keywords: Atherosclerosis; Gene expression; Hormones; Smooth muscle

Introduction

Epidemiological studies have shown that the risk for cardiovascular disease is lower in premenopausal women than in men of the same age. Hormone replacement therapy has been reported to lower the incidence of cardiovascular disease in postmenopausal women (Colditz et al., 1987; Kannel et al., 1976), although the beneficial effects of estrogen have not been confirmed in recent randomized trials (Hulley et al., 1998; Rossouw et al., 2002). A number of animal studies have also shown estrogen's anti-atherogenic effects, including amelioration of the response to vascular injury (Sullivan et al., 1995), inhibition of endothelial cell apoptosis (Sudoh et al., 2001), and nitric oxide-mediated vasodilatation (Bell et al., 1995). Estrogen receptors (ER) are expressed in the vasculature (Hodges et al., 2000; Karas et al., 1994), supporting that estrogen can exert its effect directly on the vascular wall.

Several estrogen-responsive genes, such as pS2 (Brown et al., 1984), c-fos (Weisz and Bresciani, 1988), and efp (Inoue et al., 1993), have already been identified in reproductive tissues. In the vasculature, estrogen-regulated genes without estrogen-responsive elements in their promoter region are reported (Akishita et al., 1996; Gallagher et al., 1999; Nickenig et al., 1998). The expression of c-fos (Akishita et al., 1996), angiotensin-converting enzyme (Gallagher et al., 1999), and angiotensin receptor-1 (Nickenig et al., 1998) in the aorta was downregulated by estrogen replacement in ovarietomized rats. These changes of gene expression could explain a part of atheroprotective effects of estrogen. Recently, methods for global gene analysis have been developed, and among them, the high-density oligonucleotide microarray, has come to be used as a powerful tool by many investigators. In this study, to discover new genes that might play a role in the action of estrogen, we performed microarray analysis to identify genes that are differentially expressed in the vascular wall, especially in vascular smooth muscle cells (VSMC), before and after treatment with estrogen. To confirm the results obtained from the microarray, we performed real-time polymerase chain reaction (PCR) and Northern blotting. Finally, four genes were identified as novel estrogen-regulated genes in VSMC.

Methods

Animals

Eight-week-old female Wistar rats (Oriental Yeast, Co., Ltd., Tokyo, Japan) were used in this study. They were kept individually in stainless-steel cages in a room where lighting was controlled (12 hours on, 12 hours off) and room temperature was kept at around 22°C. They were given a standard diet and water ad libitum. All the surgical procedures were performed under ether anesthesia. All of the experimental protocols were approved by the Animal Research Committee of the University of Tokyo.

Ovariectomy and E2 Implantation

Rats were randomly divided into three groups. Two groups of rats were ovariectomized and the other group of rats was sham-operated. After a two-week recovery period, one group of ovariectomized rats (OVX + E group, n = 5) underwent subcutaneous implantation of a three-week releasing pellet containing 0.5 mg 17β-estradiol (E2; Innovative Research of America). The other group of ovariectomized rats (OVX + V group, n = 5) and sham-operated rats (Sham group, n = 4) received placebo pellets. Two weeks after pellet implantation, blood samples were obtained from rats. Serum estradiol concentration was 5.6 ± 1.5 pg/ml in the Sham group (n = 4), 2.8 ± 1.0 pg/ml in the OVX + V group (n = 5), and 74.5 ± 12.1 pg/ml in the OVX + E group (n = 5). The thoracic aorta was obtained from rats after sacrifice. The endothelium was removed from the aorta by scraping with blade to ensure that the sample was mainly derived from VSMC.

High-density oligonucleotide microarray analysis

Total RNA was extracted from the aorta with Isogen (Wako Junyaku Ltd.) according to the manufacturer's instructions. One microgram of RNA isolated from the aorta of OVX + E group, OVX + V group and Sham group (n = 2, each group) rats was amplified up to approximately 100 μg cRNA and hybridized to the high-density oligonucleotide microarray (GeneChip Rat GenomeU34A; Affymetrix, Santa Clara, CA) as described previously (Ishii et al., 2000). This array contains probes interrogating approximately 7000 full-length rat genes. The intensity for each feature of the array was calculated by using Affymetrix Gene Chip version 3.3 software. The average intensity was made equal to the target intensity, which was set at 100, to reliably compare variable multiple arrays. In addition to the default parameters of the software, we added a criteria that >100 average intensity units per transcript was required for a gene to be considered "present" in the samples. Genes, with an intensity of around 1.5-fold higher or lower in the OVX + E group than in the OVX + V group, were identified.

Real-time PCR

Total RNA was treated with DNase (Progema) at 37°C for 1 h. One microgram of RNA was reverse transcribed into cDNA using Oligo dT primer (GIBCO) and an Ominiscript kit (GIBCO). Real-time PCR was carried out in an iCycler (BioRad) at 95°C for 15 min to activate HotStar Taq DNA polymerase, followed by 35 cycles of 94°C for 15 sec, 55°C for 30 sec and 72°C for 30 sec using a SYBR green assay kit (TAKARA). Amplicons were around 100 bp long. We selected the primer sets that amplified the sequences as close as possible to the 3′ coding region of the target genes. The sequences of the primers are shown in Table 1. The expression levels of each gene were normalized for glyceraldehyde-3-phosphate dehydrogenase expression.

Cell culture

VSMC were harvested from the aorta of Wistar rats by enzymatic dissociation, as previously reported (Watanabe et al., 2001). Cells were maintained in Dulbecco's modified Eagle's medium (Nikken Bio Medical Laboratory, Tokyo) supplemented with 10% fetal bovine serum (Intergen Co., Purchase, NY), penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37°C in a humidified atmosphere of 95% air and

Table 1
Primers used for quantification of mRNA levels

Accession no.	Definition	Forward primer	Reverse primer
U48247	Enigma	ttcgtctccaccaaacactg	tccctctgctagctcctgag
Z46614	Caveolin1	gcatcctctctttcctgcac	tggaatagacacggctgatg
U44948	SmLIM	taatgtggatggccttaccg	ggatgggcaggagagtgtag
AF000942	Id3a	cctcgaccttcaagtggttc	acgttcagatgagcctggtc
M17701	Glyceraldehyde-3-phosphate-dehydrogenase	cttccgtgttcctaccc	acctggtcctcagtgtagcc
M83107	SM22	tgagcaagttggtgaacagc	attgagccacctgttccatc
X06801	αSMactin	gctctggtgtgtgacaatgg	aaccatcactccctggtgtc
U50044	von Willebrand factor	agcgggtgaaatacctagcc	gcagtcagttggcctctacc

5% CO2. VSMC at 6–10 passages were used in the experiments. Cells were seeded in 10-cm-culture dishes to grow to confluence. Then, the medium was replaced with phenol red-free RPMI1640 (Sigma) containing 100 nM E2 (Sigma) or vehicle (0.1% ethanol). Twenty-four hours later, cells were washed with phosphate-buffered saline twice and homogenized immediately in Isogen reagent (Nippon Gene, Osaka, Japan).

Northern blot analysis

Twenty micrograms of total RNA from cultured VSMC were fractionated on 1.3% formaldehyde-agarose gel and transferred to nylon filters (Hybond-N, Amersham Life Science Inc.). The filters were hybridized with random-primed ^{32}P-labeled rat cDNA probes and autoradiographed. To synthesize cDNA probes, reverse transcription-PCR was performed using RNA prepared from VSMC with primers specific for each gene. The primers were synthesized according to the published rat cDNA sequences as follows: (forward/reverse)

Enigma: 5′-gccttctcagcagtcagctt-3′/5′-ttcttctggatgccaggact-3′
Caveolin-1: 5′-cgtagactccgagggacatc-3′/5′-gctcttgatgcacggtacaa-3′
Smooth muscle LIM protein (SmLIM): 5′-gaagaggtgcagtgtgatgg-3′/5′-tctggagcacttctcagcac-3′
Inhibitor of DNA binding 3a (Id3a): 5′-ggaacgtagcctagccattg-3′/5′-ttcagatgagcctggtctagc-3′.

Amplified PCR products were subcloned into a plasmid vector, pCR2.1 vector, and sequenced. An oligonucleotide probe complementary to 18S rRNA was used to confirm the equal loading of RNA. (Watanabe et al., 2001) The filters were autoradiographed, and the bands were scanned and the density was determined with Scion software (Scion image ver 3.0, Scion Corp.).

Statistical analysis

The mRNA levels calculated in real-time PCR were analyzed using one-way ANOVA. When a statistically significant effect was found, Newman-Keul's test was performed to isolate the difference between the groups. A value of $P < 0.05$ was considered significant. All data in the text and figures are expressed as mean ± SE.

Results

Screening for genes expressed differently between OVX + V and OVX + E by high-density oligonucleotide array

We first performed a global expression analysis of approximately 7000 genes using a high-density oligonucleotide microarray to identify estrogen-regulated genes in the rat aorta. Around 2000 genes were considered to be present in the aorta according to our criteria. As shown in Table 2, the expression of control GAPDH was comparable among the groups, suggesting that the microarray assay worked well. The expression of SM22 was high, whereas that of von Willebrand factor and endothelial nitric oxide synthase was below the detection level. These findings indicate that the samples were mainly derived from the medial layer of the aorta. In this screening, we identified approximately 200 genes, the expression levels of which were different between the OVX + E group and OVX + V group. We, first, checked the genes reported to be regulated by estrogen in the aorta, such as angiotensin II type 1 receptor (Nickenig et al., 1998), angiotensin converting enzyme (Gallagher et al., 1999), and c-fos (Akishita et al., 1996), and in reproductive tissues, such as progesterone receptor (May et al., 1989), c-myc (Weisz and Bresciani, 1988), and glucose-6-phosphate dehydrogenase (Korach et al., 1985). Consistent with the previous data, the intensity of angiotensin converting enzyme in OVX + E was down-regulated to nearly 50% compared to that in OVX + V. However, AT1 receptor, c-myc and progesterone receptor were not detected in aorta by high-density oligonucleotide microarray analysis probably because of the low sensitivity to these genes. Also, in sham-operated rats, the intensity of c-fos gene was at much higher level compared to that in OVX + V. The reason for a tremendous increase of c-fos expression might result from unknown stresses, because the intensity of several immediate-early genes was also increased in sham-operated rats (data not shown). The explanations for these results were that the sensitivity of probes for several genes was under the threshold, and/or that the reproducibility was not high due to small number of samples in each group (n = 2). Then, among the 200 genes, we focused on up to 20 candidate genes, which were reported to be expressed in the vasculature.

Table 2
Expression of marker genes and previously reported estrogen-regulated genes in aorta

Accession No.	Definition	Sham (Intensity)	OVX+V (Intensity)	OVX+E (Intensity)
M17701	Glyceraldehyde-3-phosphate-dehydrogenase	1278.5	1232.6	1246.0
M83107	SM22	4350.8	4487.8	4631.9
U50044	von Willebrand factor	8.7	-54.8	-19.8
AF110508	endothelial nitric oxide synthase	48.4	48.1	45.3
M90065	angiotensin II receptor	-7.5	5.1	4.2
U03734	angiotensin converting enzyme	216.6	239.9	148.3
X06769	c-fos	1800.1	307.7	231.8
S64044	progesterone receptor	61.3	31.7	39.8
X07467	glucose-6-phosphate dehydrogenase	474.0	332.1	454.2
Y00396	c-myc	44.4	36.3	33.3

Table 3
Genes with altered expression level in aorta according to DNA microarray technique

Accession no.	Definition	Sham (intensity)	OVX + V (intensity)	OVX + E (intensity)	OVX + E/OVX + V
U48247	Enigma	288.3	128.6	455.5	3.5
Z46614	Caveolin-1	674.3	329.1	694.4	2.1
U44948	SmLIM	1266.9	1260.7	2054.9	1.6
AF000942	Id3a	201.7	224.6	318.3	1.4

Confirmation of estrogen-regulated genes in aorta by real-time PCR

Next, we performed real-time PCR to examine the expression of the candidate genes obtained from the microarray. In real-time PCR, we used primers that amplified sequences different from the microarray. Subsequently, four genes, caveolin1, enigma, SmLIM and Id3a, were identified as being upregulated in the OVX + E group (Table 3 and Fig. 1). On the other hand, we could not identify any genes down-regulated in the OVX + E group in this study, so far. To exclude the possibility of the contamination with other cell types in total RNA samples we used, we compared the intensity of these four genes and markers for endothelium or VSMC in the samples between with or without endothelium obtained from intact 8-week-old male rats (n = 12) (Fig. 2). Semi-quantitative analysis by real-time PCR showed that these four genes and markers of VSMC were expressed comparably between samples with or without endothelium. In contrast, the expression of an endothelial marker, von Willebrand factor, was scanty in endothelium-denuded samples. Specific markers for adventitial fibroblasts have not been identified (Sartore et al., 2001). Therefore, we cannot exclude the contamination with adventitial fibroblasts, although the adventitial layer is very small in amount compared with smooth muscle layers.

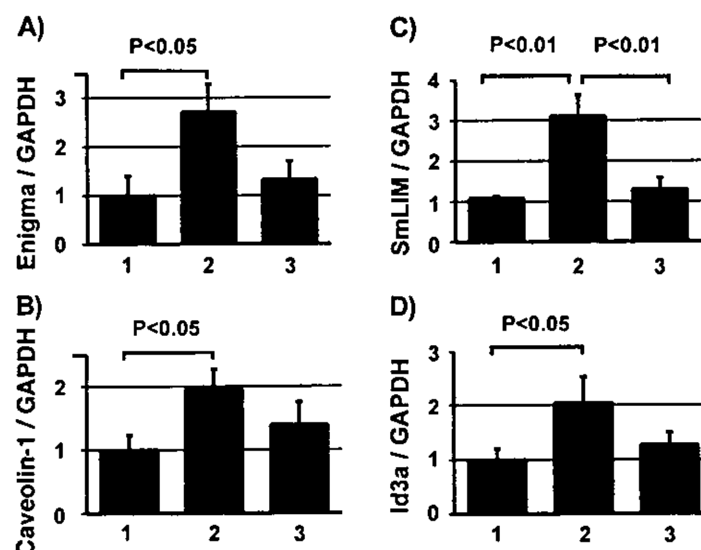

Fig. 1. Real-time PCR comparing expression of enigma, caveolin-1, SmLIM and Id3a in aortic tissue. Total RNA was obtained from the aorta of OVX + V (lane 1, n = 5), OVX + E (lane 2, n = 5), and Sham (lane 3, n = 4) groups, and reverse-transcribed into cDNA. Then, 50 ng cDNA was amplified using primers specific for each gene sequence using real-time PCR method. The starting quantities were calculated and expressed as the ratio of each gene to GAPDH. Values are shown as mean ± SE.

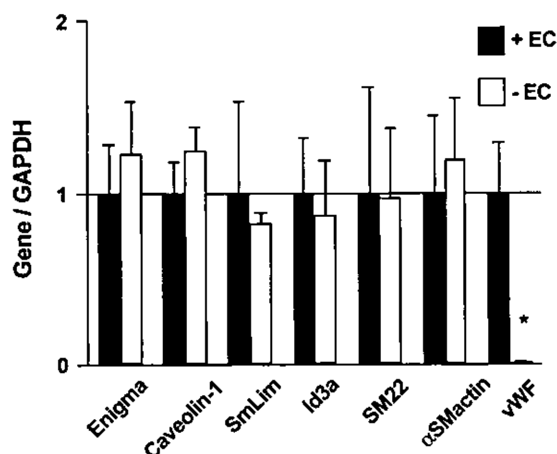

Fig. 2. The expression levels in the identified genes and maker genes in the samples with or without endothelium (EC). The aortic tissues were obtained from intact 8-week male rats, and were divided into two groups; with EC (n = 6) and without EC (n = 6). Real-time PCR was performed as described above, and the starting quantities were calculated and expressed as the ratio of each gene to GAPDH. Values are shown as the ratio of the samples with EC to that without EC and as mean ± SE. *, $p < 0.01$ vs + EC. EC; endothelium, vWF; von Willebrand factor.

E2-induced expression of genes in cultured VSMC

In order to investigate whether E2 could directly regulate the expression of these four genes, we examined their mRNA levels in cultured VSMC by Northern blot analysis. As shown in Fig. 3, treatment with E2 for 24 hours increased the mRNA levels of caveolin1, enigma, SmLIM and Id3a mRNA.

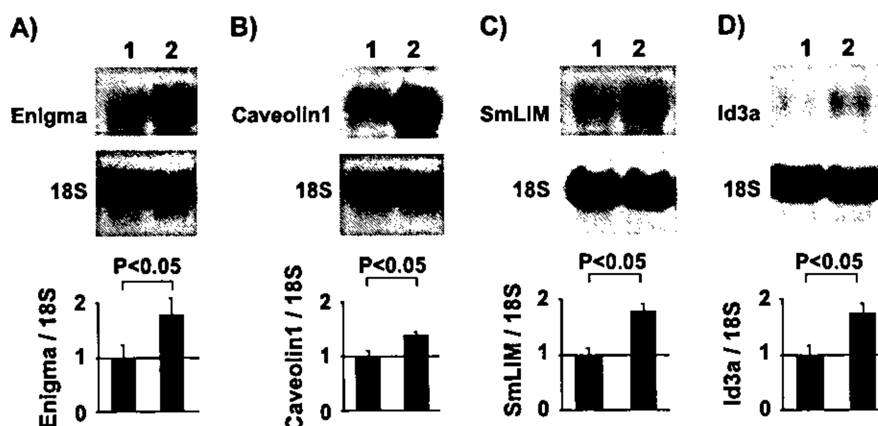

Fig. 3. Northern blot analysis of enigma, caveolin-1, SmLIM1 and Id3a in cultured VSMC. VSMC were treated with vehicle (lane 1) or 100 nmol/L E2 (lane 2) for 24 hours. Total RNA was extracted from VSMC, and 20 μg total RNA per lane was used for Northern blot analysis. The membrane was hybridized to a 32P-labeled cDNA probe specific for each gene and to an 18S probe to assess loading differences. In different sets of experiments, mRNA levels of indicated genes were measured by densitometry and expressed as the ratio of genes to 18S. Similar results were obtained in three independent experiments.

Discussion

In the present study, we screened for genes that responded to estrogen stimulation in VSMC. We newly identified genes upregulated by estrogen; enigma, SmLIM, caveolin and Id3a, in VSMC.

Caveolin-1 is one subtype of caveolins, which are principal coat proteins of caveolae (Severs, 1988). Caveolae, the flask-shaped vesicular invaginations of the plasma membrane, are present in many cell types including VSMC (Drab et al., 2001). Caveolae function in signal transduction (Okamoto et al., 1998) as well as in endocytosis and transcytosis in vesicular transport (Schnitzer et al., 1995). Mice lacking the caveolin-1 gene show impaired endothelium-dependent relaxation, contractility and maintenance of myogenic tone of the aorta through nitric oxide and Ca^{2+} signaling (Drab et al., 2001). Several studies have reported the role of caveolin-1 in estrogen-mediated signaling in vascular cells. In vascular endothelium, nitric oxide synthase is activated rapidly by estrogen following binding with ERα in caveolae (Chambliss et al., 2000). In VSMC, estrogen stimulated the binding of ERα with caveolin-1 and augmented the production of caveolin-1 through a transcriptional mechanism (Razandi et al., 2002). Consistent with this report, we showed that estrogen upregulated mRNA expression of caveolin-1 in the aorta, as well as in cultured VSMC. Taken together, estrogen-mediated upregulation of cavelin-1 might be related to the improvement of vascular function.

Two LIM protein genes and one member of the Id gene family were also identified as estrogen-regulated genes in the aorta in the present study. LIM proteins are a protein family containing the LIM motif, a double-zinc-finger structure. The LIM motif has been proposed to participate in protein-protein interactions (Dawid et al., 1995; Sanchez-Garcia and Rabbitts, 1994), and to be critical in cellular determination and differentiation (Arber and Caroni, 1996; Schmeichel and Beckerle, 1994). SmLIM, one of the LIM proteins, is expressed principally in VSMC of adult animals and is induced in VSMC during development, preceding the appearance of the smooth muscle myosin heavy chain, a sensitive indicator of VSMC differentiation (Jain et al., 1998). Moreover, SmLIM localizes in the nucleus and in actin-based filaments in the cytosol. Therefore, SmLIM is thought to coordinate cytoskeletal function and subsequently regulate cellular proliferation and differentiation (Jain et al., 1998). Another LIM protein, enigma, belongs to the PDZ-LIM protein, and is expressed abundantly in skeletal muscle as well as in non-muscle cells (Durick et al., 1998; Guy et al., 1999). The PDZ domain of enigma binds to a skeletal muscle target, the actin-binding protein, tropomyosin, suggesting that enigma is an adapter protein that directs the LIM-binding protein to actin filaments of muscle cells (Guy et al., 1999). The inhibitor of DNA binding (Id), a class of helix-loop-helix transcription factors, is known to regulate growth in many cells including VSMC (Matsumura et al., 2001; Norton et al., 1998; Olson, 1990). There are four known Id genes, Id1 to Id4. Id3a is produced by alternative splicing of the Id3 gene, resulting in inclusion of a 115-bp "coding intron", which encodes a unique 29-amino-acid carboxyl terminus of the Id3a protein (Matsumura et al., 2001). It is reported that Id3a is associated with apoptotic activity in VSMC (Matsumura et al., 2001). In contrast, another group showed that Id3 mediated angiotensin II-induced cell growth (Mueller et al., 2002); therefore, the precise role of Id3 and its splice variant, Id3a, in the vasculature, has not been determined.

There are no reports with respect to the regulation of these three genes by estrogen, not only in the vasculature but also in other organs, so our findings might imply a new understanding of mechanisms of the effects of estrogen in the vascular wall. Because SmLIM and Id3a may be associated with cell growth and differentiation, these genes might mediate the effects of estrogen on VSMC growth and differentiation. Enigma is considered to be an adaptor protein, which can connect some kinases or