Table 1. Changes in Functional Parameters According to Treatment Group

Functional Parameters	Mean ± Standard Deviation								P-Value
	Testosterone				Control				
	Baseline	3 Months	6 Months	Difference: 0 to 6 Months	Baseline	3 Months	6 Months	Difference: 0 to 6 Months	
Mini-Mental State Examination	20.2 ± 4.5	21.8 ± 4.7	22.6 ± 6.5*	2.4 ± 3.1	21.9 ± 5.3	22.0 ± 4.6	22.0 ± 4.1	0.1 ± 2.7	.03
Hasegawa Dementia Scale, Revised	17.6 ± 5.9	18.2 ± 7.1	20.6 ± 7.3*	3.0 ± 4.3	19.6 ± 5.6	20.1 ± 7.0	18.8 ± 7.7	− 0.8 ± 2.3	.02
Barthel Index	91 ± 12	89 ± 17	91 ± 15	0.5 ± 7.1	92 ± 10	91 ± 10	92 ± 7	0.4 ± 7.6	.70
Vitality Index	9.0 ± 0.9	9.3 ± 0.9	7.9 ± 1.3	− 1.1 ± 1.0	9.0 ± 1.0	9.4 ± 1.0	9.4 ± 0.9	0.4 ± 1.0	.35

P-values are based on repeated-measures analysis of variance comparing the 6-month change between the groups.
*$P < .05$ compared with baseline.

cognitive function in Japanese older men with mild to moderate cognitive decline is reported.

Eleven men with cognitive impairment, mean age 81 ± 6, receiving long-term care, were assigned to take oral testosterone undecanoate 40 mg daily for 6 months after a breakfast containing 15 to 20 g of fat. The control group of 13 men matched for age and cognitive function were followed without testosterone treatment. Cognitive function was evaluated using the Mini-Mental State Examination (MMSE) and Hasegawa Dementia Scale, Revised (HDS-R) at baseline and at 3 and 6 months. Plasma hormone levels were also measured. The institutional review board approved the study protocol, and all participants or their families gave written informed consent.

At baseline, mean total and free testosterone levels, calculated using the Vermeulen equation,[8] were 14 ± 4 nmol/L and 246 ± 47 pmol/L, respectively. There were no significant differences between the groups in age, length of education, nutritional parameters, functional parameters, or plasma hormone levels. Fasting plasma testosterone levels in the morning did not change significantly during the study, whereas the post-dose levels increased up to 30 ± 8 nmol/L 6 hours after testosterone administration, as reported previously.[9] The changes in functional parameters in each group from baseline to 6 months are shown in Table 1. At 3 months, subjects who received testosterone treatment showed a nonsignificant increase in MMSE and HDS-R scores, whereas at 6 months, cognitive scores were significantly greater than at baseline. In the control group, both cognitive scores remained unchanged. The difference between the groups was significant at 6 months. Prostate-specific antigen and liver function were unchanged, and no adverse effects were observed.

No significant changes were observed in basic activities of daily living (ADL) and ADL-related vitality in either group (Table 1), possibly because these scores were preserved in most subjects at baseline; the Barthel Index and Vitality Index[10] were 91 ± 10 (full score = 100) and 9.0 ± 1.0 (full score = 10), respectively.

This preliminary study needs to be confirmed in a randomized controlled trial with a large sample size. Nevertheless, these results indicate the effects of testosterone treatment on cognitive function in frail elderly men.

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A CASE OF SELECTIVE SEROTONIN REUPTAKE INHIBITOR-INDUCED RAPID EYE MOVEMENT BEHAVIOR DISORDER

To the Editor: Rapid eye movement (REM) sleep behavior disorder (RBD) is often seen in older patients and is characterized by a loss of normal skeletal muscle atonia during REM sleep.[1,2] As a result, the disease manifests as nocturnal motor activity consistent with the enactment of dream content, for example grabbing the bed partner in response to a dream about falling from a cliff. RBD often results in injury to the patient, bed partner, or both.[1,2]

In perhaps up to two-thirds of cases, RBD is associated with neurodegenerative disorders, most notably the alpha-synucleinopathies (Parkinson's disease, Lewy body disease, multiple systems atrophy), often antedating other manifestations of these disorders by many years.[1–4] Other cases seem to be idiopathic, although it has been suggested that various medications, notably selective serotonin reuptake inhibitors (SSRI) and other antidepressants, may commonly induce RBD.[1,4,5] In spite of this assertion, there have been few supporting case reports.[5,6] The authors recently cared for a man who clearly developed RBD as a result of SSRI treatment; the use of the SSRI for posttraumatic stress disorder (PTSD) complicated the clinical picture.

CASE REPORT

An 87-year-old male World War II veteran had been treated for PTSD with associated nightmares but no nocturnal motor activity with bupropion and lorazepam. Past medical history was significant only for essential hypertension. In 1998, after many years of treatment, sertraline was added because of increasing symptoms. Within 6 months of adding sertraline, the patient developed frequent nocturnal motor behavior consistent with the content of his dreams and nightmares, for example punching and choking his wife in the context of a dream about being in a fight. As a result, he and his wife had suffered lacerations and contusions. Other behaviors included running out of his bedroom or running into a window. Upon awakening, he was able to recall portions of the dreams but was unaware of the motor behaviors.

Trials of temazepam, zolpidem, and trazodone were ineffective in improving these behaviors. Ultimately, a diagnosis of RBD was made based on the clinical presentation. Clonazepam 1 mg at bedtime was added, which resulted in a moderate decrease in the frequency of the nocturnal motor activity, from nightly to two or three times per week. After 3 months, sertraline was slowly tapered and discontinued, which resulted in a complete cessation of all nocturnal motor behavior. He remained free of nocturnal motor activity for 5 months, until sertraline was inadvertently restarted after the loss of his wife. Within 1 month of restarting sertraline, the nocturnal motor behavior returned. There has thus far been no evidence of dementia or of parkinsonism.

This patient's clinical presentation was typical of RBD; unfortunately, his and his wife's injuries were also typical. It seems clear that his RBD was SSRI induced; it developed after sertraline was started, did not definitively improve until it was stopped, and recurred after it was inadvertently restarted, and there was no evidence of parkinsonism or dementia over the previous 12 years. Although there are few published cases of SSRI-induced overt RBD, increased electromyography activity during REM sleep has been demonstrated in patients taking SSRIs. (None of the patients were being treated for PTSD.)[7]

The relationship between RBD and PTSD is complex and not fully investigated. There is clinical and polysomnographic evidence of greater motor activity during REM sleep in patients with PTSD,[8] and greater prevalence of RBD was noted in a cohort of patients with PTSD.[9] SSRIs are effective for PTSD-related nightmares[10] but may cause RBD, clonazepam is effective for RBD[1,2,4] but not for PTSD-related nightmares,[10] and RBD is not associated with the typical diurnal symptoms of PTSD. In spite of his long history of PTSD and related nightmares, this patient had never exhibited any significant motor activity during sleep until the SSRI was started.

RBD is relatively common in geriatric practice and should be explored in any patient with nocturnal injuries or motor activity. RBD responds well to treatment, generally with clonazepam. Discontinuation of SSRIs or changing to

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ORIGINAL ARTICLE: EPIDEMIOLOGY, CLINICAL PRACTICE AND HEALTH

Effects of dehydroepiandrosterone supplementation on cognitive function and activities of daily living in older women with mild to moderate cognitive impairment

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Aim: There is little evidence that dehydroepiandrosterone (DHEA) has beneficial effects on physical and psychological functions in older women. We investigated the effect of DHEA supplementation on cognitive function and ADL in older women with cognitive impairment.

Methods: A total of 27 women aged 65–90 years (mean ± standard deviation, 83 ± 6) with mild to moderate cognitive impairment (Mini-Mental State Examination, MMSE; 10–28/30 points), receiving long-term care at a facility in Japan were enrolled. Twelve women were assigned to receive DHEA 25 mg/day p.o. for 6 months. The control group ($n = 15$) matched for age and cognitive function was followed without hormone replacement. Cognitive function was assessed by MMSE and Hasegawa Dementia Scale-Revised (HDS-R), and basic activities of daily living (ADL) by Barthel Index at baseline, 3 and 6 months. Plasma hormone levels including testosterone, DHEA, DHEA-sulfate and estradiol were also followed up.

Results: After 6 months, DHEA treatment significantly increased plasma testosterone, DHEA and DHEA-sulfate levels by 2–3-fold but not estradiol level compared to baseline. DHEA administration increased cognitive scores and maintained basic ADL score, while cognition and basic ADL deteriorated in the control group (6-month change in DHEA group vs control group; MMSE, +0.6 ± 3.2 vs −2.1 ± 2.2, $P < 0.05$; HDS-R, +2.8 ± 2.8 vs −0.3 ± 4.1, $P < 0.05$; Barthel Index, +3.7 ± 7.1 vs −2.7 ± 4.6, $P = 0.05$). Among the cognitive domains, DHEA treatment improved verbal fluency ($P < 0.05$).

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Conclusion: DHEA supplementation in older women with cognitive impairment may have beneficial effects on cognitive function and ADL. **Geriatr Gerontol Int 2010; 10: 280–287.**

Keywords: activities of daily living, cognitive function, dehydroepiandrosterone.

Introduction

Dehydroepiandrosterone (DHEA) and its sulfate (DHEA-S) are the most abundant circulating steroids mainly produced by the adrenal zona reticularis in both sexes.[1] Their circulating levels decline with advancing age,[1–4] and there has been growing public interest in DHEA supplementation to prevent age-associated physical and cognitive impairment. DHEA is considered a crucial precursor of human sex steroid biosynthesis, and to exert indirect androgenic and estrogenic effects following conversion into smaller amounts of testosterone and estradiol.[5,6] While this conversion contributes to a part of testosterone production in men, its role may be much more significant in postmenopausal women whose ovarian production of androgen and estrogen has waned. Importantly, postmenopausal women with intact ovaries continue to produce androgens; DHEA(-S), testosterone and androstenedione, while their production of estradiol is minimal.[7] However, the role of androgens in older women's health is not fully understood.

Clinical trials of the effects of estrogen replacement therapy on cognitive function have shown a lack of efficacy in postmenopausal women initiating hormone replacement therapy after the age of 65 years.[8,9] On the other hand, previous reports have suggested that DHEA may have neuroprotective effects, and the age-associated DHEA(-S) decline is associated with cognitive impairment in older women.[2,10–12] One longitudinal study observed lower DHEA-S levels in patients who subsequently developed Alzheimer's disease.[13] However, controlled trials with DHEA supplementation have failed to show beneficial effects on cognition in healthy middle-aged to older women.[14–16] In these studies, the participants were limited to those who did not have cognitive impairment; therefore, it is reasonable to hypothesize that DHEA supplementation may be effective in much older women with cognitive decline as well as lower DHEA levels.

Dehydroepiandrosterone deficiency is also considered to be involved in the development of physical frailty.[17] Clinical experience with DHEA supplementation in older women is limited, and the few clinical trials examining its effect on physical function and activity of daily living (ADL) have yielded inconsistent results.[18–20] Evidence is lacking for much older women in whom physical impairment becomes more apparent and is accompanied by an age-associated DHEA decline. In our previous study, plasma DHEA and DHEA-S levels, but not estradiol level, were independently related to higher basic ADL in older women aged 70–93 years with functional decline receiving long-term care.[21] We hypothesized that in older women, DHEA replacement could be effective for the age-related decline of physical as well as psychological function.

This study therefore examined the effect of relatively low-dose (25 mg daily) p.o. DHEA supplementation for 6 months on cognitive function and ADL in older women with cognitive impairment.

Methods

Subjects and study design

In this open, non-randomized controlled study, 27 women aged 65 years or older who attended a health service facility for the elderly (a facility that provides nursing care and rehabilitation services to elderly people with disability, Mahoroba-no-Sato, located in Nagano Prefecture, Japan) were enrolled. The participants were in a chronic stable condition and receiving Long-term Care Insurance service either for admission to the facility or day-care services. The principal inclusion criteria were mild to moderate cognitive decline; both Mini-Mental State Examination (MMSE)[22] and Hasegawa Dementia Scale-Revised (HDS-R)[23] scores were between 10 and 28. The subjects were diagnosed as having a mild cognitive impairment[24] or Alzheimer's disease according to the Diagnostic and Statistical Manual of Mental Disorders IV.[25] The participants had never been treated with hormone replacement therapy, and plasma DHEA-S concentration was less than 3.0 μmol/L. The exclusion criteria were history of stroke, extremely low ADL status (Barthel Index[26] <50), malnutrition (serum albumin <3.5 mg/dL), malignancy, acute inflammation (fever, white blood cell count >10 000/μL, or other signs of infection within 4 weeks before enrollment) and overt endocrine diseases, because these diseases may affect both plasma sex hormone levels and functions. None of the subjects were taking a cholinesterase inhibitor (donepezil hydrochloride) or glucocorticoid, opiate or hormone supplement.

Twelve women were assigned to receive DHEA capsule (25 mg/day, Athena Clinics International,

Honolulu, HI, USA) and 15 women were followed up without any additive medication. Medications that could influence cognitive function and plasma hormone levels were not changed during the study period. Outcome measures were cognitive function, ADL, plasma hormone levels, blood cell counts, blood chemical parameters and subjective adverse events. They were assessed at baseline, and after 3 and 6 months. The institutional review board of Mahoroba-no-Sato approved the study protocol, and all participants or their families gave written informed consent.

Hormone measurements

Blood samples were obtained from the participants in the morning after an overnight fast, and plasma hormone levels in addition to blood cell counts and blood chemical parameters were determined by a commercial laboratory (Health Sciences Research Institute, Yokohama, Japan). DHEA and DHEA-S were assayed using sensitive radioimmunoassays with minimum detection limits of 0.04 ng/mL (0.14 nmol/L) and 2.0 μg/dL (0.05 μmol/L), respectively. Total testosterone and estradiol were assayed using chemiluminescent immunoassays with minimum detection limits of 7 ng/dL (0.2 nmol/L) and 4 pg/mL (14.7 pmol/L), respectively. The intra-assay coefficients of variation for these measurements were less than 5%.

Cognitive function

Trained examiners administered two standardized cognitive function tests, MMSE[22] and HDS-R,[23] to assess multiple, diverse aspects of cognitive function at baseline and at the 3- and 6-month visits. Both scores range 0–30, with higher scores indicating better performance. HDS-R includes questions about the subject's age, orientation, immediate recall, serial subtraction of 7 s, reciting digits backward, recalling three words, recalling five objects and word fluency (generating names of vegetables). MMSE evaluates five aspects of cognition: (i) orientation; (ii) registration; (iii) attention and calculation; (iv) recall; and (v) comprehension of spoken language (naming objects, spoken language ability, following commands). MMSE, but not HDS-R, includes four performance tests: (i) three-stage command; (ii) reading and following a command; (iii) writing; and (iv) construction drawing). Based on the results of HDS-R and MMSE, we evaluated seven cognitive domains (points) as follows: (i) orientation (10); (ii) verbal memory (9); (iii) attention and calculation (5); (iv) visual memory (5); (v) spoken-language comprehension (9); (vi) verbal fluency (5); and (vii) performance (7).

Other functional parameters and anthropometric measures

Trained nurses and physical therapists visited the participants at the facility and performed the assessments. Basic ADL was assessed by Barthel Index,[26] mood by Geriatric Depression Scale (GDS, 15 items),[27] and ADL-related vitality by Vitality Index (10-point scale).[28] Higher GDS scores indicate a more marked self-reported depressive status, while higher Vitality Index scores indicate greater willingness.

Adverse events

Information regarding adverse events was obtained by questioning or examining the subjects. At each visit during the treatment period, all new complaints and symptoms were recorded. The safety of DHEA supplementation was assessed from the symptoms and by measuring blood chemical parameters including liver and kidney function, electrolyte levels and hematological parameters. Preexisting complaints or symptoms that increased in intensity or frequency during the treatment period also were examined.

Statistical analysis

Data were analyzed using SPSS statistical software ver. 17.0. Changes in outcome measures at 3 and 6 months were calculated by comparing the values at baseline with those at each measurement. Within each group, the significance of the change from baseline to 6 months was tested using paired Student's *t*-test. Repeated-measures ANOVA was used to test the statistical significance of the effects of DHEA versus control. Significance tests were two-sided, with an α-level of 0.05.

Results

Hormone changes and adverse effects

Characteristics and hormone levels at baseline according to treatment groups are shown in Table 1. There were no significant differences between the DHEA group and the control group in age, length of education, nutritional parameters, functional parameters and plasma hormone levels. DHEA supplementation was well tolerated, with high adherence, and there were no detectable adverse events and none of the subjects dropped out during the study. Measures of liver function, kidney function, electrolyte levels and hemoglobin level were not significantly altered by treatment with DHEA (data not shown). Body mass index remained unchanged in both groups.

Subjects in the DHEA group showed a significant increase from baseline to 3 and 6 months in levels of

Table 1 Participant characteristics at baseline

	DHEA	Control
No. of subjects	12	15
Age, years	82 ± 6 (69–90)	83 ± 6 (65–89)
Education, years	8 ± 2	8 ± 2
Nutritional parameters		
Body mass index, kg/m^2	22.0 ± 2.4 (18.8–26.4)	22.4 ± 3.2 (17.6–27.1)
Albumin, g/dL	4.4 ± 0.3 (3.7–4.9)	4.3 ± 3.2 (3.8–4.7)
Total cholesterol, mg/dL	227 ± 39 (166–294)	203 ± 22 (173–250)
Functional parameters		
MMSE	24.0 ± 4.2 (18–28)	23.4 ± 4.4 (14–28)
HDS-R	19.9 ± 5.8 (10–28)	21.7 ± 5.6 (10–28)
Barthel Index	89.6 ± 9.4 (55–100)	89.7 ± 6.4 (75–100)
Vitality Index	9.8 ± 0.6 (8–10)	9.9 ± 0.3 (9–10)
GDS	7.0 ± 4.4 (1–15)	7.0 ± 4.0 (1–13)
Hormones		
DHEA-S, μmol/L	1.8 ± 0.6 (0.7–2.4)	1.6 ± 0.8 (0.3–2.9)
DHEA, nmol/L	7.6 ± 4.7 (2.4–19.1)	6.6 ± 3.1 (2.1–11.5)
Testosterone, nmol/L	1.4 ± 0.4 (0.9–2.3)	1.3 ± 0.9 (0.2–3.8)
Estradiol, pmol/L	88 ± 52 (15–187)	70 ± 26 (45–115)

Values are shown as mean ± standard deviation (range). HDS-R, Hasegawa Dementia Scale-Revised; MMSE, Mini-Mental State Examination; GDS, Geriatric Depression Scale; DHEA-S, dehydroepiandrosterone sulfate; DHEA, dehydroepiandrosterone. There was no significant difference in each parameter between the groups.

circulating DHEA, DHEA-S and testosterone, with levels reaching approximately 2–3-fold higher than those at baseline, whereas the increase in estradiol level was not significant (Table 2). Subjects in the control group showed no significant change in hormone levels.

Changes in cognitive function and ADL

The changes in functional parameters in each group from baseline to 6 months are shown in Table 2. After 6 months, mean HDS-R score significantly improved in the DHEA group while it remained unchanged in the control group. Mean MMSE score significantly declined in the control group while it remained unchanged in the DHEA group. As a result, significant differences were found in these scores between the groups. DHEA treatment maintained Barthel Index score, whereas the score deteriorated significantly during 6 months in the control group, although the between-group difference at 6 months was not statistically significant. Regarding the components of Barthel Index, in the control group, the sum score of mobility deteriorated significantly after 6 months compared to baseline, while no significant change was observed in the sum score of self care (Table 3). Neither Vitality Index nor GDS changed significantly in both groups.

Table 4 shows the cognitive domain scores at baseline and at 3- and 6-month follow up. Among the seven cognitive domains, DHEA treatment improved verbal fluency ($P < 0.05$), resulting in a significant difference at 6 months between the groups. Verbal memory showed a non-significant trend towards improvement in the DHEA group. Performance test scores significantly declined over time in both groups. There were no differences between the groups in the scores of orientation, attention and calculation, visual memory and spoken-language comprehension.

Discussion

Daily administration of DHEA 25 mg for 6 months in elderly women with mild to moderate cognitive impairment improved cognitive function and maintained basic ADL, compared to the control group. Among the cognitive domains, DHEA significantly improved verbal fluency. At baseline, DHEA and DHEA-S levels were lower than those reported in healthy postmenopausal women in both groups,[2,4] and DHEA treatment increased DHEA, DHEA-S and testosterone levels by 2–3-fold to the mid-normal range for premenopausal

Table 2 Changes in hormone levels and functional parameters by treatment group

	DHEA				Control				*P*
	Baseline	3 months	6 months	0–6-month difference	Baseline	3 months	6 months	0–6-month difference	
Hormones									
DHEA-S, μmol/L	1.8 ± 0.6	4.5 ± 1.3*	5.6 ± 2.9*	3.8 ± 2.8	1.6 ± 0.8	1.8 ± 1.0	1.7 ± 0.8	−0.02 ± 0.4	<0.01
DHEA, nmol/L	7.6 ± 4.7	12.2 ± 4.8*	13.7 ± 7.7*	6.1 ± 8.2	6.6 ± 3.1	7.3 ± 3.7	7.4 ± 4.5	0.9 ± 2.8	0.04
Testosterone, nmol/L	1.4 ± 0.4	2.3 ± 0.7*	2.3 ± 0.8*	0.9 ± 0.8	1.4 ± 0.7	1.4 ± 0.7	1.6 ± 0.8	0.2 ± 0.5	<0.01
Estradiol, pmol/L	88 ± 52	92 ± 48	101 ± 37	13 ± 51	70 ± 26	68 ± 20	67 ± 42	−4.0 ± 38	0.17
Functional parameters									
MMSE	24.0 ± 4.2	24.1 ± 4.6	24.6 ± 4.3	0.6 ± 3.2	23.4 ± 4.4	23.1 ± 5.4	21.3 ± 5.0**	−2.1 ± 2.2	0.04
HDS-R	19.9 ± 5.8	20.5 ± 7.3	22.7 ± 6.3**	2.8 ± 2.8	21.7 ± 5.6	22.1 ± 5.6	21.3 ± 6.4	−0.3 ± 4.1	0.04
Barthel Index	89.6 ± 9.4	92.7 ± 6.5	93.3 ± 6.8	3.7 ± 7.1	89.7 ± 6.4	86.9 ± 7.2	87.0 ± 6.7*	−2.7 ± 4.6	0.04
Vitality Index	9.8 ± 0.6	9.7 ± 0.5	9.7 ± 0.7	−0.1 ± 1.0	9.9 ± 0.3	9.8 ± 0.5	9.7 ± 1.0	−0.3 ± 1.0	0.80
GDS	7.0 ± 4.4	6.2 ± 3.4	6.6 ± 3.7	−0.4 ± 1.7	7.0 ± 4.0	8.3 ± 3.9	7.5 ± 3.5	0.5 ± 3.3	0.60

Values are shown as mean ± standard deviation (range). *P*-values are for repeated-measure ANOVA over all three time points. DHEA, dehydroepiandrosterone; HDS-R, Hasegawa Dementia Scale-Revised; MMSE, Mini-Mental State Examination; GDS, Geriatric Depression Scale; DHEA-S, dehydroepiandrosterone sulfate; DHEA, dehydroepiandrosterone. **$P < 0.01$ compared to baseline, *$P < 0.05$ compared to baseline.

women.[2] No detectable adverse effects were observed throughout the study.

According to the previous trials, DHEA supplementation of 50 mg or more daily does not provide beneficial effects on cognition in healthy middle-aged to elderly women without cognitive impairment.[14–16] However, in a small-scale randomized double-blind placebo-controlled study, DHEA transiently improved cognition (after 3 months) in subjects with Alzheimer's disease while the improvement was not significant at 6 months.[29] Preliminary analysis of the small number of subjects in the present study suggested that DHEA treatment was no less effective in subjects with low baseline cognitive function than those with higher cognitive function (data not shown). Whether the effects of DHEA might be influenced by baseline cognitive function should be further investigated.

It is noteworthy that the 6-month effect of donepezil hydrochloride (5 or 10 mg), the only cholinesterase inhibitor used in Japan, in patients with Alzheimer's disease ranged from no change to less than 1 point improvement in MMSE score,[29–33] which is not so different from the effect of DHEA observed in the present study.

In the present study, not only the participants' cognitive function was impaired, but baseline plasma DHEA(-S) level was also low compared to that in postmenopausal or perimenopausal women.[2,4,10] Regarding DHEA-S levels, according to a report in which healthy pre- and postmenopausal women were studied, DHEA-S levels in women aged 35–44 years and 45–55 years were as follows: 4.31 ± 2.11, 3.90 (mean ± standard deviation) and 3.42 ± 2.01 μmol/L.[2] In this study, DHEA-S was measured using chemiluminescent enzyme immunometric assay; although the measurements by this method and those by radioimmunoassay have been reported to be comparable. In our study, DHEA treatment increased DHEA-S levels to the mid-normal range for premenopausal women.[2] Also, the subjects with lower baseline DHEA-S levels showed non-significant trend towards more improvement in cognitive scores (data not shown). Thus, future studies are needed to explore whether the effects of DHEA might be influenced by baseline DHEA levels.

Because the DHEA receptor has not been identified, DHEA may act after conversion to testosterone and subsequently estradiol through estrogen receptors and androgen receptors, both of which are found in the hippocampus and frontal lobes and subserve verbal memory and working memory in women.[34,35] Further, hippocampal volume and perfusion have been shown to correlate with serum DHEA-S level in demented patients.[36,37] It has also been suggested that estrogenic and androgenic derivatives of DHEA might have different effects on cognitive functions.[38] However, the mechanism by which DHEA improves cognitive

Table 3 Changes in mobility and self-care scores in Barthel Index during the study

Domains (points)	Mean ± SD Baseline	3 months	6 months	Change (0–6 months)	*P*
Mobility (55)					
DHEA	46.9 ± 9.2	48.2 ± 6.0	49.2 ± 5.2	2.3 ± 5.4	0.01
Control	47.5 ± 5.4	46.2 ± 5.5	45.0 ± 4.3*	−3.7 ± 3.9	
Self care (45)					
DHEA	42.7 ± 6.1	44.5 ± 1.5	43.1 ± 2.5	0.4 ± 6.9	0.96
Control	41.8 ± 4.2	42.5 ± 3.4	41.2 ± 4.3	0.7 ± 3.2	

Mobility is the sum score of five domains: (i) transfer (moving from a bed to a wheelchair and back); (ii) walking on a level surface; (iii) propelling a wheel chair; (iv) ascending and descending stairs; and (v) bathing and toilet use. Self care includes feeding, grooming, dressing, bowels and bladder. *P*-values are for repeated-measure ANOVA over all three time points. $^{*}P < 0.05$ compared to baseline. SD, standard deviation.

Table 4 Changes in cognitive domain scores during study

Domains (points)	Mean ± SD Baseline	3 months	6 months	Change (0–6 months)	*P*
Orientation (10)					
DHEA	8.3 ± 1.9	8.0 ± 2.7	7.5 ± 3.0	−0.1 ± 1.2	0.28
Control	8.3 ± 1.9	8.0 ± 2.8	7.5 ± 2.9	−0.7 ± 1.7	
Verbal memory (9)					
DHEA	5.7 ± 2.1	6.5 ± 2.3	6.7 ± 2.5†	1.0 ± 1.9	0.79
Control	6.5 ± 1.7	7.5 ± 1.8	7.0 ± 1.9	0.5 ± 1.7	
Attention and calculation (5)					
DHEA	2.3 ± 1.9	2.8 ± 2.0	2.7 ± 1.8	0 ± 2.3	0.79
Control	2.0 ± 1.7	1.9 ± 1.2	1.8 ± 1.5	−0.5 ± 1.4	
Visual memory (5)					
DHEA	3.6 ± 0.9	3.6 ± 1.3	3.8 ± 1.2	0.3 ± 1.1	0.91
Control	3.6 ± 1.3	3.9 ± 0.9	3.9 ± 1.0	0.5 ± 1.1	
Language comprehension (9)					
DHEA	8.5 ± 0.8	7.8 ± 2.5	8.7 ± 0.7	0.1 ± 0.3	0.12
Control	8.5 ± 0.8	8.5 ± 0.8	8.4 ± 1.1	−0.1 ± 0.9	
Verbal fluency (5)					
DHEA	2.8 ± 3.3	2.5 ± 2.0	4.3 ± 1.1*	1.5 ± 1.7	0.01
Control	3.2 ± 1.9	3.8 ± 1.6	3.3 ± 1.9	0.1 ± 2.1	
Performance (7)					
DHEA	5.7 ± 0.7	5.5 ± 0.7	4.8 ± 0.4**	−0.8 ± 0.6	0.36
Control	5.6 ± 0.6	5.1 ± 0.6	4.5 ± 0.9**	−1.1 ± 0.8	

Change refers to score change during 0–6 months for each parameter in each treatment group. *P*-values are for repeated-measure ANOVA over all three time points. DHEA, dehydroepiandrosterone. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{\dagger}P < 0.1$ vs baseline. SD, standard deviation.

function is unknown. In the present study, plasma estradiol level was not significantly increased after DHEA treatment, implying that its beneficial effects on cognition might be androgen-dependent. Unfortunately, free testosterone levels were not measured, because they were considered to be undetectable in many cases in older women. In addition, sex hormone-binding globulin (SHBG) measurement was not available; however, it has been reported that DHEA 50 mg treatment for 3 months in postmenopausal women did not significantly change SHBG levels,[39] suggesting that the change in SHBG-bound hormone levels after DHEA treatment might be minimal. Given the local aromatization of androgen to estradiol in the brain, the effect of DHEA on cognition might be indirect, complex and heterogeneous. The molecular mechanism underlying the association

between DHEA and cognitive function needs to be clarified, and active forms of testosterone and estradiol should also be examined to investigate whether they would change after DHEA administration.

In our previous study, plasma DHEA and DHEA-S levels were independently related to higher basic ADL in older women aged 70–93 years with functional decline,[21] and other reports have shown a correlation between DHEA level and muscle mass, strength and physical performance.[40,41] In the present study, DHEA treatment maintained the Barthel Index score, while the score deteriorated significantly in the control group. Regarding body composition and strength, DHEA administration in postmenopausal older women aged up to 80 years did not alter body composition, physical performance or strength.[18–20] However, in one small-scale open-label trial, DHEA treatment for 4 weeks improved ADL in three out of seven patients (both men and women) with multi-infarct dementia.[42] All these studies are preliminary, and large-scale and long-term studies are required to ascertain whether DHEA could have a beneficial effect on ADL in older women.

In the present study, no effect of DHEA on depressive mood or vitality was observed, consistent with most clinical trials in older women.[15,43,44] This might be attributable to the participants' relatively low depressive status and high vitality status, namely, ceiling effects.

The limitations of our study should be acknowledged. First, this study was neither blinded nor randomized. Second, the number of participants was too small to confirm the results. Thus, results need to be confirmed by large-scale randomized trials to exclude possible selection bias. Third, considering the sensitivity and accuracy, a standard test like the Alzheimer's Disease Assessment Scale should be used in clinical trials to ascertain the effect of DHEA. Finally, our study duration was 6 months so it does not provide any information on the effects of longer-term DHEA supplementation.

In summary, this small study showed that supplementation of DHEA 25 mg for 6 months to older women with mild to moderate cognitive impairment improved cognitive scores and maintained basic ADL. The results should be confirmed in large-scale randomized trials.

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DHEA attenuates PDGF-induced phenotypic proliferation of vascular smooth muscle A7r5 cells through redox regulation ☆

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ABSTRACT

It is known that dehydroepiandrosterone (DHEA) inhibits a phenotypic switch in vascular smooth muscle cells (VSMC) induced by platelet-derived growth factor (PDGF)-BB. However, the mechanism behind the effect of DHEA on VSMC is not clear. Previously we reported that low molecular weight-protein tyrosine phosphatase (LMW-PTP) dephosphorylates PDGF receptor (PDGFR)-β via a redox-dependent mechanism involving glutathione (GSH)/glutaredoxin (GRX)1. Here we demonstrate that the redox regulation of PDGFR-β is involved in the effect of DHEA on VSMC. DHEA suppressed the PDGF-BB-dependent phosphorylation of PDGFR-β. As expected, DHEA increased the levels of GSH and GRX1, and the GSH/GRX1 system maintained the redox state of LMW-PTP. Down-regulation of the expression of LMW-PTP using siRNA restored the suppression of PDGFR-β-phosphorylation by DHEA. A promoter analysis of GRX1 and γ-glutamylcysteine synthetase (γ-GCS), a rate-limiting enzyme of GSH synthesis, showed that DHEA up-regulated the transcriptional activity at the peroxisome proliferator-activated receptor (PPAR) response element, suggesting PPARα plays a role in the induction of GRX1 and γ-GCS expression by DHEA. In conclusion, the redox regulation of PDGFR-β is involved in the suppressive effect of DHEA on VSMC proliferation through the up-regulation of GSH/GRX system.

1. Introduction

Vascular smooth muscle cells (VSMC), the contractile component of blood vessels, play a critical role in the pathogenesis of atherosclerosis. VSMC express a set of smooth muscle-specific genes, which are characteristic of their contractile, differentiated phenotype [1]. VSMC undergo phenotypic modulation in response to environmental signals. Accelerated migration, proliferation, and production of extracellular matrix components by phenotypically modulated VSMC play a central role in the development of atherosclerotic lesions [1].

Platelet-derived growth factors (PDGFs) bind to two cell-surface receptor-tyrosine kinases, PDGF receptor (PDGFR) α and β [2]. PDGF-dependent activation of receptors causes a mitogenic signal transduction through the phosphorylation of specific tyrosines in the receptors [2–4].

Dehydroepiandrosterone (DHEA) and the sulfated prohormone of DHEA circulate at higher plasma concentrations than any other steroids. Both the occurrence and the clinical manifestation of coronary atherosclerosis have been inversely correlated with plasma levels of DHEA or DHEA sulfate [5]. DHEA has a wide variety of beneficial biological and physiological effects on the prevention of cardiovascular disease [6].

The redox status of sulfhydryl groups in proteins plays an important role in the regulation of cellular functions such as the synthesis and folding of proteins and regulation of the structure and activity of enzymes, receptors, and transcription factors [7]. Glutaredoxin (GRX), a glutathione (GSH)-dependent oxidoreductase, catalyzes the reduction of protein disulfide via a disulfide exchange reaction [8]. Previously, we reported that GRX1 plays an important role in regulating PDGF-BB-dependent signals through down-regulation of the tyrosine phosphorylation of PDGFR-β [3]. The GSH/GRX1 system suppresses the PDGF-BB-induced tyrosine phosphorylation of PDGFR-β, resulting in suppression of the PDGF-BB-dependent cell proliferation. Furthermore, we found a novel regulatory mechanism for PDGF-BB signaling involving the redox-dependent regulation of low molecular weight-protein tyrosine phosphatase (LMW-PTP) by GRX1 in a GSH-dependent manner [3]. Recently, we also reported

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that estradiol potentiates GSH/GRX1 redox potential in cardiomyocytes through up-regulation of the gene expression of γ-glutamylcysteine synthetase, the rate-limiting enzyme of GSH synthesis and GRX1 [9]. Then, we were interested in whether DHEA has any effects on the GSH/GRX1 system in VSMC exposed to PDGF-BB. In the present study, we demonstrate that DHEA attenuates PDGF-BB-induced VSMC proliferation and phenotypic modulation. Importantly, we show that DHEA increases the expression of GRX and GSH synthesis. This increase in GSH/GRX1 redox potential stimulates the LMW-PTP to down-regulate the activity for tyrosine phosphorylation of PDGFR-β.

2. Materials and methods

2.1. Reagents

Rabbit antibodies against PDGFR-β and phospho (Tyr-751)-PDGFR-β were obtained from Cell Signaling Technology. PDGF-BB, GSH, GSSG, NADPH, 3-(4,5-dimethyl-thiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), flutamide, and *p*-nitrophenyl phosphate were from Sigma Chemical Co. DHEA, hydrogen peroxide, and dithiothreitol (DTT) were from Wako Pure Chemicals (Osaka, Japan). Normal goat, rabbit, and mouse IgG were from Sigma Chemical Co. 4-Acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (AMS) was purchased from Molecular Probes. ICI182,780 was from Tocris (Ballwin, MO).

2.2. Cell culture and proliferation

Rat embryonic thoracic aorta smooth muscle-derived A7r5 cells were obtained from American Type Culture Collection (Manassas, VA) and cultured as described [10]. Briefly, cells were incubated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. After the attainment of confluency (70–80%), the cells were incubated in serum-free DMEM containing 0.2% bovine serum albumin for 20–24 h. The proliferation of cultured cells was evaluated by measuring attached live cells photometrically after staining with crystal violet. A7r5 cells incubated in the presence or absence of DHEA were placed in 100 μl of medium/well in 96-well plates and cultured in medium containing 0.2% BSA with or without 0.5 nM PDGF-BB for specific periods. Then the cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.5), washed, and stained with 0.01% crystal violet at room temperature for 20 min. Each well was extensively washed with water and dried. The stained cells were lysed by adding 100 μl of lysis buffer A (10% SDS and 0.1 N HCl), and the cell number was then estimated photometrically by measuring the absorbance at 570 nm using a microplate reader.

2.3. Purification of recombinant LMW-PTP and generation of antibody against LMW-PTP

LMW-PTP was purified with the glutathione *S*-transferase (GST) gene fusion system (Amersham Biosciences) according to the manufacturer's instructions. In brief, *Escherichia coli* strain BL21 cells were transformed with pGEX6p-LMW-PTP, and protein expression was induced by isopropyl β-D-1-thiogalactopyranoside (IPTG). GST-fused LMW-PTP (GST-LMW-PTP) was affinity purified from cell lysates using glutathione–Sepharose 4B (Amersham Biosciences), and then digested with PreScission protease. The cleaved GST was removed from the glutathione–Sepharose 4B, and LMW-PTP was purified. The LMW-PTP was used to immunize rabbits to generate anti-LMW-PTP antibodies, and also used for experiments concerning the redox regulation of LMW-PTP as described below.

2.4. Immunoblot analysis

Cultured cells were harvested and lysed for 20 min at 4 °C in lysis buffer. The supernatants obtained by centrifugation of the lysates at 8000*g* for 15 min were used in subsequent experiments. Protein concentrations were determined using a BCA assay kit (Pierce). Protein samples were electrophoresed on 10%, 12.5% or 15% SDS–polyacrylamide gels under reducing conditions, except for thiol-modified protein samples. The proteins in the gels were transferred onto nitrocellulose membranes, and were incubated with horseradish peroxidase-conjugated anti-IgG antibodies. Proteins in the membranes were visualized using the enhanced chemiluminescence detection kit (Amersham Biosciences) according to the manufacturer's instructions.

2.5. Determination of redox states

The redox states of proteins were assessed by modifying free thiol with AMS [11]. Briefly, after incubation with or without PDGF-BB, cell lysates or proteins were treated with trichloroacetic acid at a final concentration of 7.5% to denature and precipitate the proteins as well as to avoid any subsequent redox reactions. The protein precipitates were collected by centrifugation at 12,000*g* for 10 min at 4 °C. The pellets were rinsed in acetone and centrifuged twice, then, dissolved in a buffer containing 50 mM Tris–HCl (pH 7.4), 1% SDS and 15 mM AMS. Proteins were then separated by 10% SDS–PAGE without using any reducing agents and blotted to nitrocellulose membranes. Proteins in the membranes were treated with 5% (w/v) nonfat dry milk and 0.1% Tween 20 in TBS solution for 1 h at room temperature, then further kept overnight at 4 °C for visualization by immunoblotting as described above.

2.6. Determination of cellular glutathione levels

GSH and glutathione disulfide (GSSG) levels were measured as described previously [9] using a Total Glutathione Quantification Kit (Dojindo Molecular Technologies, Inc., MD) according to the manufacturer's directions.

2.7. Generation of luciferase reporter constructs

A 2.0-kb fragment of the human GRX1 gene promoter (−2023 to −22) was amplified by PCR using Pfu turbo DNA polymerase (Stratagene). The primers used were /5′-GGA CTG AGT GAG AGG CAG ACA ATA GTC TCC-3′/ as a forward primer, and /5′-CGG GAA GAA TCC TCA GTT GCA GGT ATT GCT TGG-3′/ as a reverse primer. The PCR product was subcloned into pUC18 to obtain pUC18-pro-GRX. PUC18-pro-GRX was digested with HindIII, and the resulting fragment containing the promoter region from −2023 to −22 was inserted into the HindIII site of the reporter vector pGL3-Basic (Stratagene) to give pGL3-pro-GRX. To generate a deleted form of the luciferase reporter construct (pGL3-pro-GRX-del), pGL3-pro-GRX was digested with KpnI and PvuII (Takara Biomedicals). Site-directed mutagenesis for luciferase vectors was performed with pGL3-pro-GRX (−2023 to −22) as a template by using a QuickChange site-directed mutagenesis kit (Stratagene). The oligonucleotides used were; peroxisome proliferator-activated receptor (PPAR) response element (PPRE)-like 1 forward (5′-GGT CAG GAT ACC TAG CTA AAT ttT CAT TTG GTG AXA TAG AGG CCA TG-3′), and PPRE-like 1 reverse (5′-CAT GGC CTC TAT GTC ACC AAA TGA aaA TTT AGC TAG GTA TCC TGA CC-3′). The nucleotide sequence was confirmed by sequencing with an ALFexpress II system (Amersham Biosciences). We constructed a 50-bp chimeric promoter with two copies of synthesized fragment of the human γ-GCS heavy subunit gene promoter (−10,625 to −10,613 bp, GenBank Accession No. AL033397) containing a PPRE-like domain; AGATCACAGGTCA. It was annealed using a forward sequence

(5′-gac ggt acc AGA TCA CAG GTC ATT GAT AAG ATC ACA GGT Cag tgg agc tc-3′) and a reverse sequence (5′-gag ctc cac TGA CCT GTG ATC TTA TCA ATG ACC TGT GAT CTg gta ccg tc-3′). The annealed product was subcloned into pUC18 to obtain pUC18-pro-γ-GCS. pUC18-pro-γ-GCS was digested with KpnI and SacI. The resulting fragment containing the promoter region (−10625 to −10613) was inserted into the HindIII site of the reporter vector pGL3-Basic to give pGL3-pro-γ-GCS. To generate a deleted version of the luciferase reporter construct (pGL3-pro-γ-GCS-del), pGL3-pro-γ-GCS was digested with KpnI and PvuII. Site-directed mutagenesis for luciferase vectors was performed with pGL3-pro-γ-GCS as a template.

2.8. Luciferase activity assay

Each vector was introduced into A7r5 cells by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The luciferase activity was assayed with cellular extracts by using a luciferase reporter assay system (Promega).

2.9. Quantitative RT-PCR

Quantitative RT-PCR was performed using the One Step SYBR® RT-PCR kit (Perfect Real Time, TAKARA BIO INC. Japan) according to the manufacturer's directions. After the RT-PCR using Mx3000P (Stratagene), the products were analyzed using SMxProTM Software version 3.00 (Stratagene). A 764-base-pair (bp) DNA γ-GCS heavy subunit cDNA was obtained by digesting a fragment (bp 865–1628) with PstI. The 330-bp oligonucleotides for GRX1 (rat GRX sequence, Accession No. AF167981) were obtained using as a forward primer, 5′-GCA TGG CTC AGG AGT TTG TGA ACT GCA AGA TTC AG-3′, and as a reverse primer, 5′-CCT TTC ATA ACT GCA GAG CTC CAA TCT GCT TCA GC-3′. The 547-bp oligonucleotides for γ-GCS (rat γ-GCS sequence, BC081702) were obtained using 5′-CCT CTG GAG ACC AGA GTAT GGG AGT TAC-3′, and 5′-GCA GAT AGT GGC CAA CTG GTC ATA AAG G-3′. The 410-bp oligonucleotides for rat β-actin (BC063166) were obtained using, 5′-GAG CTA TGA GCT GCC TGA CG-3′, and 5′-AGC ATT TGC GGT GCA CGA TG-3′.

2.10. RNA interference and transfections

Double-stranded small interfering RNAs (siRNAs) corresponding to rat GRX1 DNA sequences (GenBank Accession No. NM-022278) (5′-ACU GCA AGA UUC AGU CUG GdTdT-3′ [siRNA-GRX-1] and 5′-AAC GUG GUC UCC UGG AAU UdTdT-3′ [siRNA-GRX-2]), and to rat LMW-PTP DNA sequences (NM-021262) (5′-CAC AUU GCA CGG CAG AUU AdTdT-3′ [siRNA-LMW-PTP-1] and 5′-UGA GAG AUC UGA AUA GAA AdTdT-3′ [siRNA-LMW-PTP-2]) were synthesized and annealed by Samchully Pharm Co., Ltd., Korea. siRNAs were transfected into the cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol with a final siRNA concentration of 100 nM.

2.11. Statistical analysis

Data were presented as the mean ± SD. Differences were examined by using ANOVA (StatView software). A value of $p < 0.05$ was considered significant.

3. Results and discussion

3.1. DHEA suppresses PDGF-induced proliferation of A7r5 cells

The effect of DHEA on PDGF-induced cell proliferation was examined in A7r5 cells. Serum-starved A7r5 cells were cultured with or without 100 nM DHEA for 18 h, then in medium containing 0.2% FBS with or without 2 nM PDGF-BB. As shown in Fig. 1A, cell proliferation induced by PDGF-BB was suppressed by pretreatment with 100 nM DHEA. DHEA at 100 and 200 nM suppressed the PDGF-BB-induced proliferation in a dose-dependent manner (Fig. 1B). The data indicate that DHEA suppresses the PDGF-induced proliferation of A7r5 cells, consistent with the report by Williams et al. [12], in which the precise mechanism of the effect of DHEA on the PDGF-BB-induced proliferation of VSMC was not clear.

3.2. Suppression of VSMC marker genes by PDGF-BB is restored by DHEA

Next, the effect of DHEA on the expression of VSMC marker genes was examined. As VSMC marker genes, SMα-actin and SM22α were estimated by the quantitative RT-PCR method. After incubation with or without 100 nM DHEA for 18 h, A7r5 cells were treated with 2 nM PDGF-BB for 24 h. As shown in Fig. 1C, the expression of SM22α and SMα-actin was suppressed by treatment with PDGF-BB, whereas the suppressive effect of PDGF-BB on the VSMC phenotype was restored by DHEA. Taken together, DHEA attenuates the PDGF-induced proliferation and phenotypic switch in VSMC, suggesting that DHEA has some effects on the PDGF-BB-dependent cell signaling. Then, PDGF-BB-induced phosphorylation of PDGFR-β was examined.

3.3. DHEA down-regulates the PDGF-BB-dependent phosphorylation of PDGFR-β

PDGFR-β-mediated signals are particularly important for vascular remodeling and neointima formation [13]. As shown in Fig. 2A and B, A7r5 cells expressed abundant PDGFR-β. Then, phosphorylation of PDGFR-β was estimated by immunoblot analysis using rabbit antibodies against PDGFR-β and phosphor-(Tyr-751)-PDGFR-β. PDGF-BB-induced phosphorylation of PDGFR-β was observed at 5–30 min, with a peak at 5–10 min (lanes 2–3). Pretreatment with 100 nM DHEA suppressed the phosphorylation of PDGFR-β (lanes 6–7). The suppression of the phosphorylation of PDGFR-β by DHEA was dose-dependent (25–250 nM, data not shown). Then, the possible suppression of PDGFR-β-mediated signaling was examined.

3.4. DHEA induces the expression of GRX1 and γ-GCS mRNA and increases the level of GSH

PDGFR-mediated signaling is regulated by many factors. These factors are involved in the generation of reactive oxygen species and redox regulation [3]. We were interested in the role of the redox regulation of PDGFR-β by DHEA. The effect of DHEA on the mRNA expression of redox-related proteins, and the levels of GSH and oxidized glutathione (GSSG) were analyzed. A7r5 cells incubated with or without 100 nM DHEA for 18 h were treated with 2 nM PDGF-BB for 12 h. The expression was expressed as the relative intensity compared to the control. Treatment with DHEA increased GRX1 mRNA and γ-GCS, a rate-limiting enzyme for GSH synthesis (Fig. 2C). The level of GSH was increased by DHEA, while the level of GSSG was not changed by DHEA, resulting in the high GSH/GSSG ratio (Fig. 2D). These results indicate that DHEA increases the expression of GRX1 and γ-GCS to elevate the GSH/GRX1 redox potential as well as the GSH/GSSG ratio.

3.5. DHEA-dependent promoter activity of GRX1 and γ-GCS gene is regulated by PPARα

DHEA up-regulates the transcriptional activity mediated by PPARα [14,15]. This mechanism involves up-regulation of the expression of PPARα mRNA by DHEA [15]. To investigate the transcriptional regulation of GRX1 and γ-GCS by DHEA via the

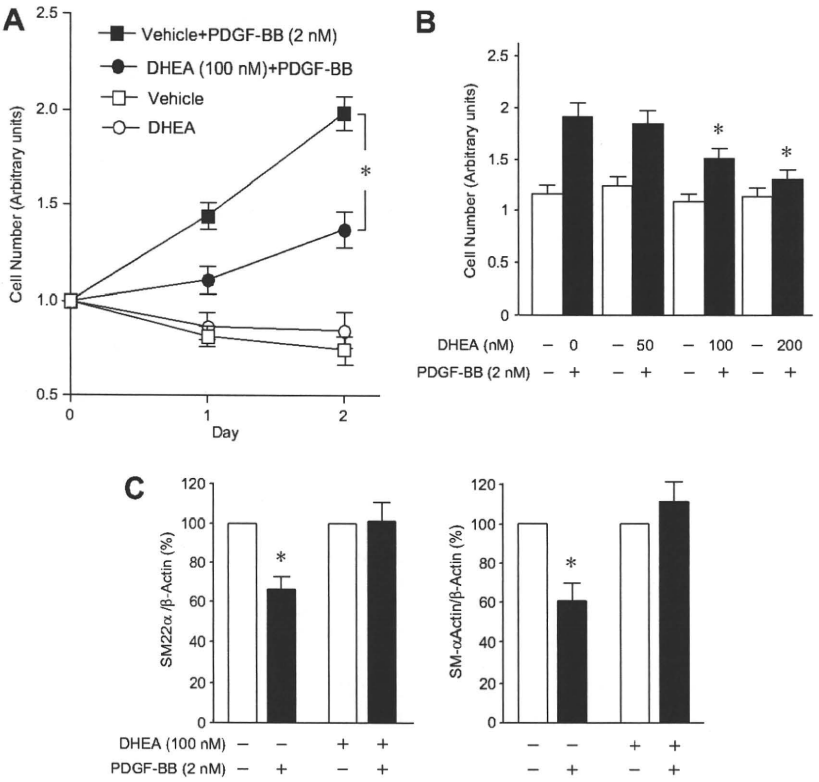

Fig. 1. Effect of DHEA on VSMC proliferation and expression of VSMC marker genes. Time-dependent (A) and dose-dependent (B) effects of DHEA on PDGF-BB-induced proliferation were estimated. (C) The expression of SM22α and SMα-actin was estimated by the quantitative RT-PCR method as described in Section 2. After incubation of A7r5 cells with or without 100 nM DHEA for 18 h, the cells were treated with 2 nM PDGF-BB for 24 h. Each value represents the mean for three independent experiments, and the SD was always within 10% of the mean. $^*p < 0.05$ compared with the control vehicle-treated cells.

PPARα-binding domain, a luciferase vector containing PPRE-like domain was constructed and introduced into A7r5 cells. The luciferase activity of the cells treated with DHEA for 18 h showed a 1.8-fold increase, but was almost lost when the PPRE-like site was deleted or mutated (Fig. 3A). Deletion of EpRE-like 2 or SP1 had no apparent effect on the DHEA-induced up-regulation of the luciferase activity (data not shown). Similarly, the promoter region of the γ-GCS heavy subunit containing a PPRE-like site was inserted into a luciferase vector. The luciferase activity of the cells treated with DHEA was stimulated by 1.6-fold, but was lost when the PPRE-like site was mutated (Fig. 3B). The results suggest that PPARα plays a role in the DHEA-induced up-regulation of the expression of GRX1 and γ-GCS.

3.6. DHEA maintained the redox state of LMW-PTP

PDGFR is a receptor-type tyrosine kinase, the activation of which is regulated by PDGF-dependent autophosphorylation and dephosphorylation by protein tyrosine phosphatases (PTPs). A number of tyrosine residues are phosphorylated in the cytosolic domain of PDGFR, leading to a site-specific recruitment of signal transduction molecules [4]. PTPs such as LMW-PTP are implicated in the control of PDGFR phosphorylation [16]. LMW-PTP is an 18-kDa enzyme that is widely expressed [17]. We focused on LMW-PTP because its redox-dependent regulation and the role in PDGF-BB/PDGFR signaling have been studied extensively [4]. Oxidative stress generated by PDGF-BB/PDGFR-β-mediated signaling causes the oxidization of LMW-PTP to form dithiothreitol-reducible high molecular weight oligomers, and the generation of peroxide is involved in the downregulation of LMW-PTP activity by PDGF-BB, leading to the activation of PDGFR-BB-dependent signaling pathways [3]. Fig. 4A shows that a decrease in the reduced form of LMW-PTP (the active form) was observed on treatment with PDGF-BB for 30 min (lane 2). The decrease in levels of the reduced form caused by PDGF-BB was similar to that observed on treatment with hydrogen peroxide (lane 5). Pretreatment of cells with DHEA for 18 h maintained the reduced form of LMW-PTP (lane 4). In this experiment, oligomerized LMW-PTP (the inactive form) was not detectable for unknown reason, and LMW-PTP-specific phosphatase activity was not determined. Together with our previous report using synthesized LMW-PTP and GSH/GRX1 redox system [3], the data suggest that DHEA maintained the redox state of LMW-PTP regulated by the GSH/GRX1 system.

3.7. Transfection of siRNAs for GRX1 and LMW-PTP abolishes the effect of DHEA on PDGF-BB-induced phosphorylation of PDGFR-β

To down-regulate the expression of GRX1 and LMW-PTP, specific siRNA (100 nM) were introduced into A7r5 cells. siRNAs bearing scrambled sequences were used as the control. At 48 h post-transfection, cells were serum-starved for 6 h, then stimulated with 2 nM PDGF-BB. Compared to the PDGF-BB-induced phosphorylation of PDGFR-β in the cells transfected with siRNA bearing scrambled sequences (Fig. 4B, lanes 2–4), the transfection of siRNA for GRX1 further stimulated the PDGF-BB-induced phosphorylation of PDGFR-β (lanes 5–8). Similarly, the cells transfected with the specific siRNA (100 nM) for LMW-PTP for 48 h showed an increase in PDGF-BB-induced phosphorylation of PDGFR-β (lanes 9–12). The results confirm that (i) DHEA increases the GSH/GRX1 redox potential, (ii) the GSH/GRX1 system is necessary to regulate the phosphorylation of PDGFR-β, (iii) the activity of LMW-PTP

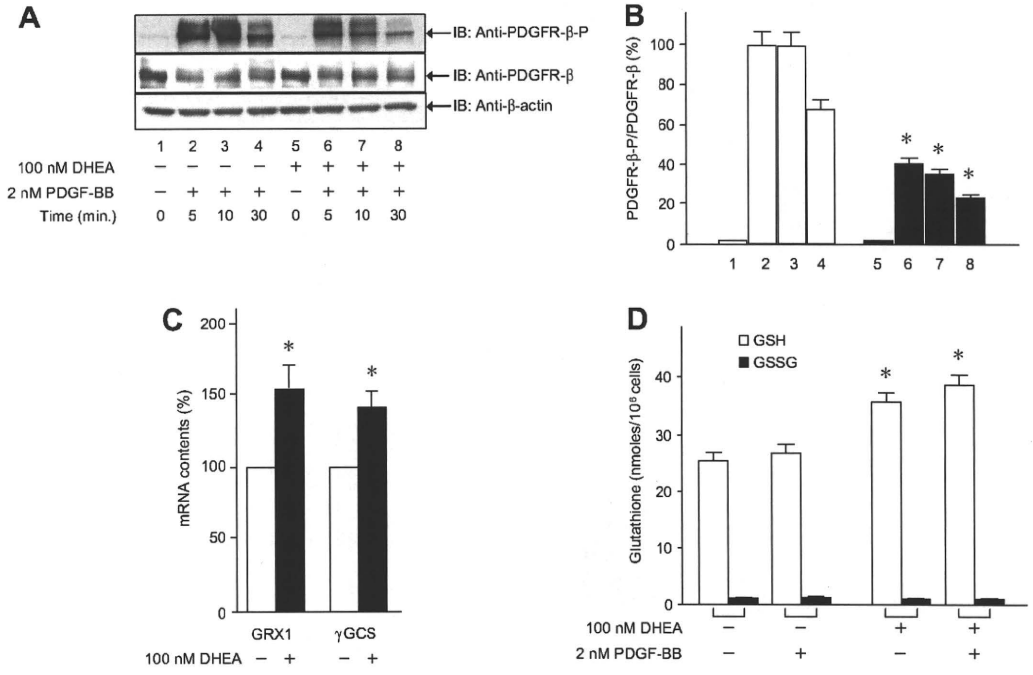

Fig. 2. DHEA down-regulates the PDGF-BB-dependent phosphorylation of PDGFR-β (A, B), and up-regulates the mRNA expression of GRX1 and γ-GCS (C) and GSH levels (D). A7r5 cells were serum-starved for 24 h. After pretreatment with 100 nM DHEA for 18 h, the cells were stimulated with 2 nM PDGF-BB for the periods indicated. (A) The phosphorylation status of PDGFR-β was examined by immunoblot analysis. (B) The intensity of the bands was estimated densitometrically, and the phosphorylation rate is expressed as the relative intensity of phosphorylated PDGFR-β to PDGFR-β protein. (C) mRNA expression of GRX1 and γ-GCS heavy subunit was analyzed by quantitative RT-PCR. The expression was expressed as relative intensity compared to the control. (D) Levels of GSH and GSSG were estimated using a Total Glutathione Quantification Kit and were expressed as relative intensity compared to the control. Each value represents the mean for three independent experiments, and the SD was always within 10% of the mean. $^*p < 0.05$ compared with the control vehicle-treated cells.

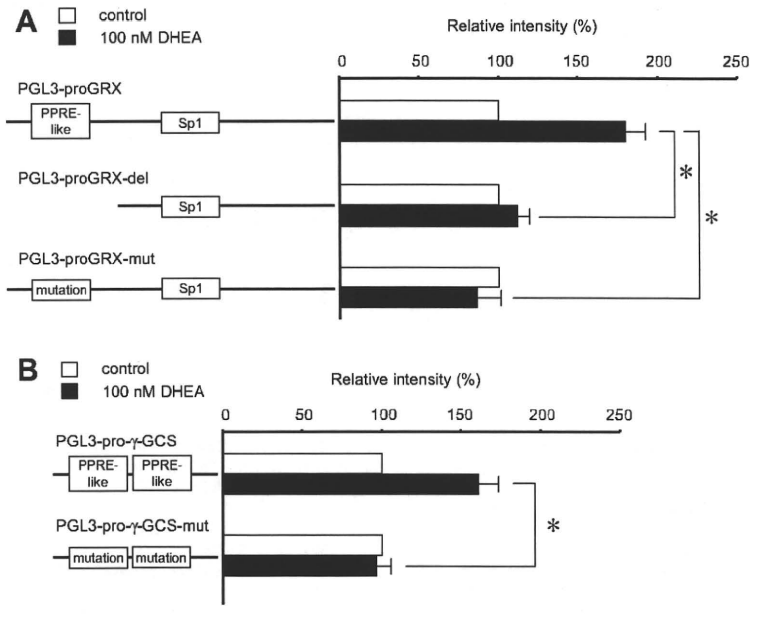

Fig. 3. PPRE-like element is important for the DHEA-dependent induction of the GRX1 and γ-GCS promoter in A7r5 cells. The transcriptional regulation of GRX1 and γ-GCS by DHEA was examined. The cells were transiently transfected with the GRX1 promoter-luciferase gene fusion plasmids (A) or the chimeric γ-GCS promoter-luciferase gene (B). After the transfection, luciferase activity was assayed with cellular extracts as described in Section 2.

maintained by DHEA regulates the phosphorylation, of PDGFR-β and (iv) the LMW-PTP-dependent dephosphorylation is regulated by the GSH/GRX1 system. To know whether the DHEA-dependent suppression of the PDGF-BB-induced phosphorylation of PDGFR-β is mediated through specific receptors for androgen or estradiol, the effect of antagonists against androgen receptor (flutamide) and estrogen receptor α/β (ICI182,780) was examined. It was found that the antagonists had no apparent effect on the DHEA-induced

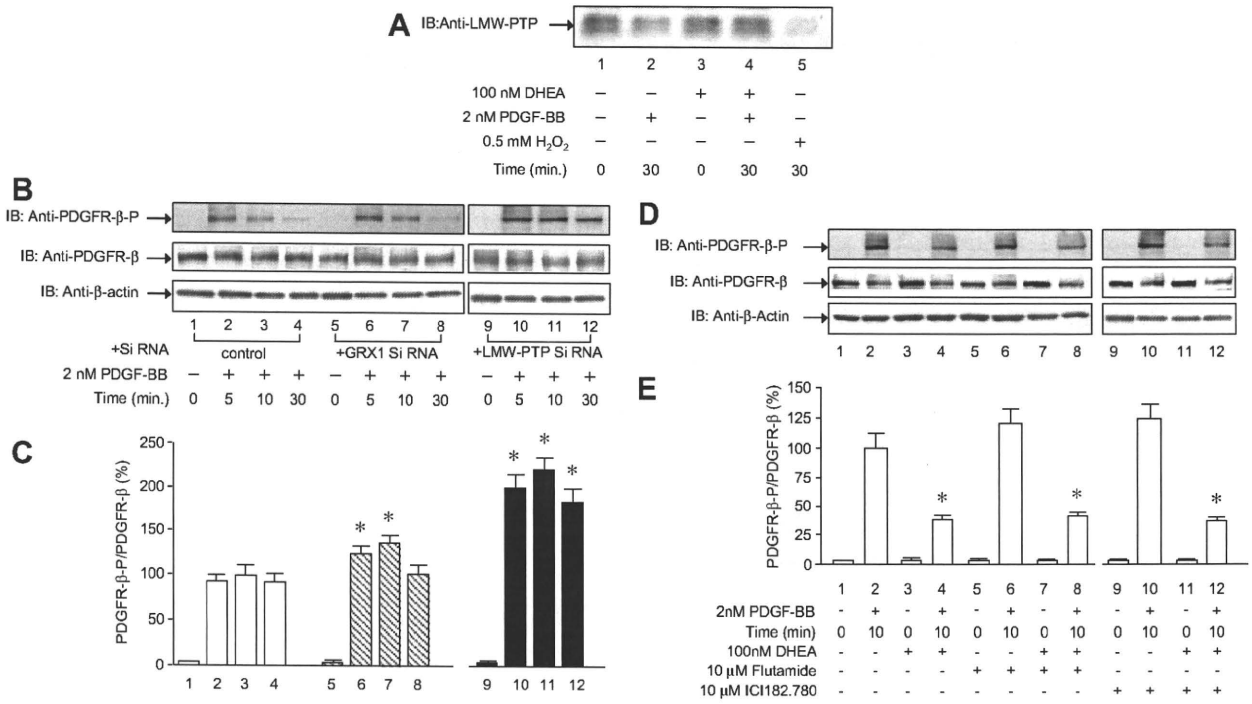

Fig. 4. The role of LMW-PTP, GRX1 and sex hormone receptors in the effects of DHEA on PDGF-BB-induced phosphorylation of PDGFR-β. (A) Effect of DHEA on the redox state of LMW-PTP was estimated. The redox state of proteins was assessed by modifying free thiol with AMS as described in Section 2. (B, C) To down-regulate the expression of GRX1, a specific siRNA for GRX1 (100 nM) was introduced into A7r5 cells. siRNA bearing a scrambled sequence was used as the control. Similarly, specific siRNA for LMW-PTP (100 nM) was introduced into the cells. (D) Effects of an androgen receptor antagonist (flutamide) and an estrogen receptor α/β antagonist (ICI182,780) on DHEA-induced suppression of PDGF-BB-dependent phosphorylation of PDGFR-β were examined. (E) The intensity of the bands was estimated densitometrically, and the phosphorylation rate is expressed as the relative intensity of phosphorylated PDGFR-β to PDGFR-β protein.

suppression of the phosphorylation (Fig. 4D, lane 4 vs. lane 8 and lane 12). The results are consistent with the previous report that the inhibitory effect of DHEA on PDGF-BB-induced proliferation of VSMC is independent of the androgen receptor and estrogen receptor [12].

In summary, this study showed that DHEA inhibits PDGFR-β phosphorylation that leads to proliferation and phenotypic changes of VSMC, and that the transcriptional control of the GSH/GRX level and the redox state of LMW-PTP may account, at least in part, for the beneficial effects of DHEA on VSMC.

Acknowledgments

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ORIGINAL ARTICLE

Association of low testosterone with metabolic syndrome and its components in middle-aged Japanese men

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Epidemiological studies have shown that low testosterone is associated with metabolic syndrome (MetS) in Caucasian men. We investigated whether testosterone level is related to the prevalence of MetS in middle-aged Japanese men. A cross-sectional survey was conducted in 194 men aged 30–64 years (49 ± 9). Blood sampling was performed in the morning after a 12-h fast, and the relationship between plasma hormone and MetS was analyzed. Low total testosterone was associated with MetS according to the Japanese criteria (HRs of 2.02 by quartile of testosterone; 95% CI=1.43–2.87) and the International Diabetes Federation criteria (HRs of 1.68 by quartile of testosterone; 95% CI=1.25–2.25). Age-adjusted regression analyses revealed that testosterone was significantly related to the MetS parameters of obesity (β=−0.365 and −0.343 for waist circumference and body mass index, respectively), hypertension (β=−0.278 and −0.157 for systolic and diastolic blood pressure, respectively), dyslipidemia (β=−0.242 and 0.228 for triglycerides and high-density lipoprotein cholesterol, respectively), insulin resistance (β=−0.253 and −0.333 for fasting plasma glucose and homeostasis model assessment of insulin resistance, respectively) and adiponectin (β=0.216). Inclusion of waist circumference into the model largely weakened the association of testosterone with other metabolic risk factors. In contrast, high estradiol was associated with MetS and its parameters, mostly attributing to the positive correlation between estradiol and obesity. Dehydroepiandrosterone sulfate was not associated with MetS or its parameters. These results suggest that low testosterone is associated with MetS and its parameters in middle-aged Japanese men. The association between estradiol and MetS needs further investigation.
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Keywords: androgen; estrogen; insulin resistance; obesity; sex hormone

INTRODUCTION

There is growing awareness that metabolic syndrome (MetS) is one of the most important threats to public health because of its association with type 2 diabetes mellitus, cardiovascular disease and mortality.[1–3] In men, it is well established that endogenous androgens decline with advancing age,[4] and low testosterone levels have been associated with insulin resistance,[5] type 2 diabetes,[6,7] hypertension[8] and increased cardiovascular and all-cause mortality.[9,10] Moreover, men with low testosterone are likely to have more components of MetS in cross-sectional studies,[11–13] and longitudinal studies show that lower total testosterone predicts higher frequency of MetS.[14,15] These data were mostly from studies with Caucasian men in western countries. Regarding Japanese men, one study showed that testosterone was positively correlated with plasma adiponectin.[16] However, there are no reports showing a relationship between testosterone and MetS or its components in Japanese men.

Recently, we reported that low testosterone is an independent determinant of endothelial dysfunction in middle-aged men[17] and is a predictor of cardiovascular events in men with coronary risk factors,[18] suggesting a link between testosterone and cardiovascular pathology. Given these findings, this study investigated the relationship of endogenous testosterone with MetS in middle-aged Japanese men.

METHODS

Subjects

Enrollment screening included consecutive, apparently healthy male subjects aged 30–64 years who underwent medical examinations at either our department or at two clinics located in Tokyo. After exclusion of subjects who met the exclusion criteria, 194 subjects (104 from our department and 90 from the clinics) were enrolled. Exclusion criteria included history of cardiovascular disease (stroke, coronary heart disease, congestive heart failure and peripheral arterial disease), malignancy or overt endocrine disease or use of steroid hormones, because these conditions may influence plasma sex hormones and/or the components of MetS. Other exclusion criteria were diabetic subjects

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on insulin injection or hypoglycemic agent drugs or with hemoglobin A1c >8%, and subjects on β-blockers[19] or fibrates. History, physical examination and laboratory tests were performed for all subjects. Of the included subjects, 23% (n=44) were taking anti-hypertensive drugs (angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, calcium channel blockers and diuretics), and 22% were taking statins. Each subject gave written, informed consent before study enrollment. The study protocol was approved by the ethics committee of the Graduate School of Medicine at the University of Tokyo.

Assays of metabolic risk factors and plasma hormones

Clinical information was collected at baseline when each patient attended the initial medical examination. Blood sampling and measurement of height, weight, waist circumference and blood pressure were performed in the morning after a 12-h overnight fast. Blood pressure was measured at least twice using an automated, digital electrosphygmomanometer (Omron Healthcare, Kyoto, Japan) on the non-dominant arm in a sitting position, and the average was used for statistical analysis.

Serum total cholesterol and triglyceride were measured enzymatically, and serum high-density lipoprotein (HDL) cholesterol was measured by the heparin-$Ca^{2+}Ni^{2+}$ precipitation method. Low-density lipoprotein cholesterol was determined using the Friedewald formula or the direct, liquid, selective detergent method when triglycerides were >400 mg per 100 ml. Plasma glucose was assayed by the glucose oxidase method, and hemoglobin A1c was measured by high-performance liquid chromatography. Plasma total testosterone, dehydroepiandrosterone sulfate and estradiol were determined using sensitive radioimmunoassays. Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as fasting insulin ($\mu IU ml^{-1}$)×fasting plasma glucose (mg per 100 ml)/405. Patients with a fasting plasma glucose >140 mg per 100 ml were excluded from the HOMA-IR calculation because of a lack of data reliability. Serum adiponectin was measured using an enzyme-linked immunosorbent assay (Human Adiponectin ELISA kit, Otsuka Pharmaceutical, Tokyo, Japan). These assays were performed by a commercial laboratory (SRL, Tokyo, Japan). The intra-assay coefficients of variation for the measurements were <5%.

Definition of MetS

We applied both the Japanese criteria[20] and the International Diabetes Federation (IDF) criteria for Japanese ethnicity[21] for the diagnosis of MetS. In the Japanese criteria, MetS was diagnosed when waist circumference ≥85 cm and two or more of the following three components were present: (1) HDL cholesterol <40 mg per 100 ml and/or triglyceride ≥150 mg per 100 ml; (2) systolic blood pressure ≥130 mm Hg and/or diastolic blood pressure ≥85 mm Hg and (3) fasting plasma glucose ≥110 mg per 100 ml. Subjects taking anti-hypertensive medications were considered hypertensive for statistical purposes.

In the IDF criteria for Japanese ethnicity, MetS was diagnosed when waist circumference ≥85 cm and two or more of the following four components were present: (1) HDL cholesterol <40 mg per 100 ml; (2) triglyceride ≥150 mg per 100 ml; (3) systolic blood pressure ≥130 mm Hg and/or diastolic blood pressure ≥85 mm Hg and (4) fasting plasma glucose ≥100 mg per 100 ml. Subjects taking anti-hypertensive medications were considered hypertensive for statistical purposes.

Data analysis

Values are expressed as the mean±s.d. in the text unless otherwise stated. Pearson's simple correlation coefficients were calculated between plasma hormones and the number of MetS components. Differences between the quartile groups of sex hormones were analyzed using one-factor ANOVA followed by the Newman–Keuls' test. Logistic regression analysis was performed to determine the association of sex hormones with the diagnosis of MetS. Furthermore, multiple regression analysis was performed to determine the association between sex hormones and metabolic risk factors for MetS. A value of $P<0.05$ was considered statistically significant. The data were analyzed using SPSS (Version 17.0, SPSS, Chicago, IL, USA).

RESULTS

Sex hormones and MetS criteria

Characteristics of the study subjects are shown in Table 1. Twenty-three and 32% of the subjects were diagnosed with MetS according to the Japanese criteria and the IDF criteria, respectively. The prevalence is comparable with that reported in middle-aged Japanese men.[22,23]

As plasma total testosterone was negatively correlated with the number of MetS components (Figure 1a), the association of testosterone with MetS was analyzed by quartile of testosterone. As shown in Figure 2a, lower testosterone was associated with a step-wise increase in the number of MetS components. Age-adjusted logistic regression analysis revealed that the hazard ratios for MetS diagnosis by quartile decline of testosterone were 2.02 (95% CI=1.43–2.87) and 1.68 (95% CI=1.25–2.25) according to the Japanese criteria and the IDF criteria, respectively.

Interestingly, plasma estradiol was positively correlated with the number of MetS components (R=0.285, $P<0.001$); therefore, the association with MetS was also analyzed by quartile of estradiol. As shown in Figure 2b, higher estradiol was associated with a step-wise increase in the number of MetS components. Age-adjusted logistic regression analysis revealed that the hazard ratios for MetS diagnosis by quartile increment of estradiol were 1.48 (95% CI=1.06–2.06) and 1.63 (95% CI=1.20–2.21) according to the Japanese criteria and the IDF criteria, respectively. Dehydroepiandrosterone sulfate was not associated with MetS components or diagnosis (data not shown).

Table 1 Characteristics of study subjects (N=194)

Age (years)	49±9	[30–64]
Body mass index (kg m^{-2})	25.2±4.0	[17.3–41.9]
Waist circumference (cm)	87±10	[69–125]
Hip circumference (cm)	96±7	[80–125]
Waist/hip ratio	0.94±0.06	[0.78–1.09]
Systolic blood pressure (mm Hg)	126±14	[95–183]
Diastolic blood pressure (mm Hg)	79±11	[50–128]
Triglycerides (mg per 100 ml)	162±135	[32–880]
HDL cholesterol (mg per 100 ml)	54±16	[26–110]
Free fatty acids (mEq l^{-1})	0.53±0.28	[0.08–2.08]
LDL cholesterol (mg per 100 ml)	128±29	[54–213]
Fasting plasma glucose (mg per 100 ml)	98±13	[76–158]
Hemoglobin A1c (%)	5.2±0.6	[4.0–8.0]
Insulin ($\mu U ml^{-1}$)	6.7±4.0	[1.0–21.2]
HOMA-IR	1.64±1.04	[0.21–5.50]
Total testosterone (nmol l^{-1})	19.1±6.2	[4.6–38.2]
DHEA-S (μmol l^{-1})	5.89±2.37	[1.12–12.0]
Estradiol (pmol l^{-1})	92.5±43.7	[18.4–216.6]
Metabolic syndrome (MetS) and its components		
MetS (Japanese criteria), n (%)		44 (23)
MetS (IDF criteria), n (%)		62 (32)
Waist circumference ≥85 cm, n (%)		110 (56)
High blood pressure, n (%)		89 (46)
HDL cholesterol <40 mg per 100 ml, n (%)		34 (18)
Triglycerides ≥150 mg per 100 ml, n (%)		79 (41)
Fasting plasma glucose ≥110 mg per 100 ml, n (%)		23 (12)
Fasting plasma glucose ≥100 mg per 100 ml, n (%)		73 (38)

Abbreviations: DHEA-S, dehydroepiandrosterone sulfate; HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment of insulin resistance; IDF, International Diabetes Federation; LDL, low-density lipoprotein.
Values are expressed as the mean±s.d. (range). High blood pressure was defined if subjects showed systolic blood pressure ≥130 mm Hg and/or diastolic blood pressure ≥85 mm Hg, or were taking antihypertensive medications.

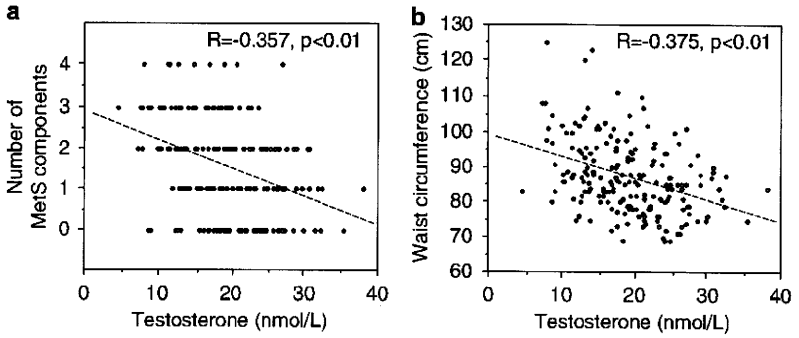

Figure 1 Scattergrams and regression lines (dotted lines) showing the correlation between testosterone and the number of metabolic syndrome (MetS) components (**a**) or waist circumference (**b**).

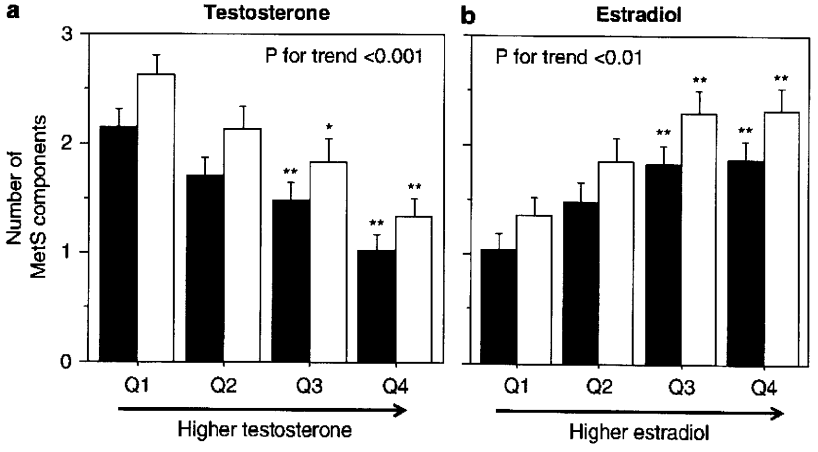

Figure 2 Number of metabolic syndrome (MetS) components according to quartiles of plasma testosterone (**a**) and estradiol (**b**). MetS components were defined according to the Japanese criteria (closed bars) and the IDF criteria for Japanese ethnicity (open bars). Values are expressed as the mean ± s.e.m. $^{*}P<0.05$, $^{**}P<0.01$ *vs.* Q1. Cut offs of the quartiles were 14.1, 18.7 and 23.4 nmol l^{-1} (405, 540 and 674 ng per 100 ml) for testosterone, and 55, 101 and 125 pmol l^{-1} (15.0, 27.5 and 34.0 pg ml^{-1}) for estradiol.

Sex hormones and metabolic risk factors

The associations of plasma sex hormones with each of the metabolic risk factors were analyzed. As shown in Table 2, the unadjusted model shows that testosterone was significantly related to parameters of MetS except for diastolic blood pressure. Testosterone was not related to low-density lipoprotein cholesterol, but this parameter is not included in the definitions of MetS used here. Adjustment for age did not considerably influence the results of the regression analysis, but the association between testosterone and diastolic blood pressure became significant after adjustment for age. In contrast, inclusion of waist circumference into the model weakened the association of testosterone with metabolic risk factors. As a result, systolic blood pressure, triglycerides, fasting plasma glucose and HOMA-IR were significantly related to testosterone. The significant association for diastolic blood pressure, HDL cholesterol, free fatty acids, hemoglobin A1c, insulin and adiponectin were attenuated after adjustment for age and waist circumference. Adjustment for body mass index or waist/hip ratio instead of waist circumference showed similar results (data not shown).

As shown in Table 3, estradiol showed weaker association than testosterone with parameters of MetS, but was significantly related to body mass index, waist circumference, systolic blood pressure, HDL

Table 2 Multiple regression analysis determining the impact of plasma testosterone on metabolic risk factors

	Unadjusted	*Age adjusted*	*Age+waist adjusted*
Body mass index	−0.376*	−0.343*	ND
Waist circumference	−0.378*	−0.365*	ND
Waist/hip ratio	−0.353*	−0.384*	ND
Systolic blood pressure	−0.230**	−0.278*	−0.169***
Diastolic blood pressure	−0.114	−0.157***	−0.098
Triglycerides	−0.247*	−0.242*	−0.182***
HDL cholesterol	0.252*	0.228**	0.065
Free fatty acids	−0.208**	−0.209**	−0.137
LDL cholesterol	−0.054	−0.056	−0.020
Fasting plasma glucose	−0.231**	−0.253**	−0.228**
Hemoglobin A1c	−0.166***	−0.220**	−0.137
Insulin	−0.331*	−0.307*	−0.129
HOMA-IR	−0.349*	−0.333*	−0.159***
Adiponectin	0.222**	0.216**	0.046

Abbreviations: HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment of insulin resistance; LDL, low-density lipoprotein; ND, not determined.
Regression coefficients with plasma testosterone as an independent variable and each of risk factors as a dependent variable are shown. Age and/or waist circumference were included in multiple regression models as indicated. $^{*}P<0.001$, $^{**}P<0.01$, $^{***}P<0.05$.

Table 3 Multiple regression analysis determining the impact of plasma estradiol on metabolic risk factors

	Unadjusted	*Age adjusted*	*Age+waist adjusted*
Body mass index	0.279*	0.260*	ND
Waist circumference	0.346*	0.338*	ND
Waist/hip ratio	0.102	0.082	ND
Systolic blood pressure	0.133	0.158**	0.042
Diastolic blood pressure	0.036	0.058	−0.002
Triglycerides	0.105	0.094	−0.012
HDL cholesterol	−0.207***	−0.193***	−0.040
Free fatty acids	0.087	0.091	0.049
LDL cholesterol	−0.056	−0.056	−0.094
Fasting plasma glucose	0.130	0.141	0.095
Hemoglobin A1c	0.040	0.067	−0.030
Insulin	0.240***	0.228***	0.038
HOMA-IR	0.250***	0.243***	0.060
Adiponectin	−0.267*	−0.262*	−0.114

Abbreviations: HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment of insulin resistance; LDL, low-density lipoprotein; ND, not determined.
Regression coefficients with plasma estradiol as an independent variable and each of risk factors as a dependent variable are shown. Age and/or waist circumference were included in multiple regression models as indicated. *$P<0.001$, **$P<0.05$, ***$P<0.01$.

cholesterol, insulin, HOMA-IR and adiponectin after adjustment for age. Further adjustment for waist circumference, body mass index or waist/hip ratio (Table 3 and data not shown) eliminated the significant associations between estradiol and these metabolic parameters. Dehydroepiandrosterone sulfate was not significantly related to parameters of MetS in unadjusted or adjusted analyses (data not shown).

DISCUSSION

In this study, cross-sectional analysis of 194 middle-aged Japanese men showed that low testosterone is positively related to MetS, MetS components and additional metabolic risk factors. Adjustment for obesity parameters such as waist circumference, body mass index and waist/hip ratio greatly diminished the association, but low testosterone retained weak associations with some metabolic risk factors including systolic blood pressure, triglycerides, fasting plasma glucose and HOMA-IR. Taken together, results in this statistical model suggest that abdominal obesity is an important contributor to the association between low testosterone and MetS, but additional factors may also impact testosterone. To our knowledge, this is the first report showing the significant association between low testosterone and MetS in Japanese men.

Several mechanisms have been suggested for the causal relationship between low testosterone and abdominal obesity. Activation of the lipoprotein lipase and lipolysis[24] may explain the effect of testosterone on adipose tissue. Many studies including a medium-sized, randomized controlled trial[25] and a meta-analysis[26] showed the inverse effect of testosterone on adiposity. Conversely, it has been reported that men with MetS are prone to hypogonadism.[27] This finding might be due to elevated leptin levels that interfere with gonadotropin-stimulated androgen production[28] and to increased aromatase activity in adipose tissue that leads to higher circulating estradiol and suppression of testosterone production by negative feedback.[29] These findings suggest a bi-directional causal relationship between low testosterone and obesity.

After adjustment for waist circumference, testosterone was weakly but significantly related to some metabolic risk factors including systolic blood pressure, triglycerides, fasting plasma glucose and HOMA-IR, which is consistent with earlier reports.[5,6,8,12] Testosterone is likely to be involved in the pathogenesis of MetS, irrespective of obesity. For example, testosterone increases the hepatic production of apolipoprotein A-1 and consequently increases HDL cholesterol,[30] improves insulin sensitivity and increases muscle strength.[31] There was no significant correlation between age and testosterone ($R=0.114$, $P=0.12$). This result may be because the cohort was limited to middle-aged men (30–64 years old) . However, age was included in the multivariate analyses in this study, because it is well established that testosterone declines with age.[4]

The positive association found between testosterone and adiponectin is in agreement with earlier reports.[16,32,33] However, the direct action of testosterone on adiponectin production/secretion might be different from these findings, because testosterone decreases adiponectin secretion in mice and in adipocytes.[34,35] Accordingly, abdominal obesity may underlie the positive correlation between testosterone and adiponectin in men.

In this study, estradiol was associated positively with MetS and its components, consistent with an earlier report.[12] This relationship may be independent of testosterone because estradiol was not correlated with testosterone by simple regression analysis ($R=-0.019$, $P=0.80$), and the inclusion of both testosterone and estradiol into the multiple regression model as covariates did not influence the association of each other with MetS parameters (data not shown). The relationship between estradiol and MetS might be attributed to increased aromatase activity and subsequent elevation of circulating estradiol in obese subjects.[29] Increased estradiol may subsequently suppress pituitary function,[29] and lead to a further decrease in testosterone. Comprehensive assessment of sex hormone, gonadotropin and components of MetS reveal a causal relationship. Unfortunately, we could not measure gonadotropin because of limited plasma. Further investigation is needed to address the mechanistic and pathophysiological interactions between sex hormones and MetS.

There are some limitations to our study. First, the cross-sectional design does not clarify the causal relationship between sex hormones and MetS. As there may be bi-directional causalities as mentioned above, longitudinal follow-up studies and hormone replacement studies should be performed in Japanese populations. Second, active forms of testosterone such as bioavailable and calculated free testosterone were not measured. A direct assay of bioavailable testosterone or of sex hormone-binding globulin (required for free testosterone calculation) was not available for the study. Third, the potential influence of medications on the measured parameters cannot be denied, although the exclusion of subjects on statins ($n=40$) or anti-hypertensive drugs ($n=44$) did not seriously affect the association of testosterone with waist circumference (statins, $R=-0.304$, $P<0.01$; anti-hypertensives, $R=-0.337$, $P<0.01$) and the number of MetS components (statins, $R=-0.274$, $P<0.01$; anti-hypertensives, $R=-0.278$, $P<0.01$). Fourth, because the sample size ($n=194$) is relatively small, the finding needs to be confirmed in a larger cohort.

In summary, this study suggests that low testosterone is associated with MetS and its parameters in middle-aged Japanese men. We also found a positive but weaker association between estradiol and MetS. These associations were largely attenuated by adjustment for waist circumference. Our results reinforce the need to address the causal relationship and pathophysiological interactions between sex hormones and MetS.

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