

A Relative High Dose of Vitamin E Does Not Attenuate Unweighting-Induced Oxidative Stress and Ubiquitination in Rat Skeletal Muscle

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Abstract We previously reported that intragastric administration of cysteine could be beneficial to prevent unweighting-induced ubiquitination and degradation of muscle protein in association with redox regulation [Ikemoto et al., *Biol. Chem.*, 383 (2002), 715–721]. In this study, we investigated whether vitamin E, another potent antioxidative nutrient, also had beneficial effects on the muscle protein catabolism. However, daily intragastric supplementation of 1.5 or 15 mg/rat of α -tocopherol did not prevent weight loss of hindlimb skeletal muscle in tail-suspended rats. To elucidate the reason for the non-effectiveness of vitamin E, we further examined concentrations of oxidative stress markers, ubiquitination of muscle proteins and fragmentation of myosin heavy chain in gastrocnemius muscle of rats daily treated with 15 mg of α -tocopherol. Unexpectedly, vitamin E increased concentrations of glutathione disulfide and thiobarbituric acid-reactive substance and decreased glutathione level in the muscle, compared with those of vehicle treatment, indicating that vitamin E enhanced unweighting-induced oxidative stress in skeletal muscle. The vitamin E supplementation did not suppress the ubiquitination of muscle proteins and fragmentation of myosin heavy chain caused by tail-suspension. Our results suggest that supplementation of a relative high dose of vitamin E could not inhibit ubiquitin-dependent degradation of muscle protein in tail-suspended rats possibly due to its prooxidant action. *J Physiol Anthropol* 21 (5): 257–263, 2002 <http://www.jstage.jst.go.jp/en>

Introduction

Skeletal muscles are vulnerable to rapid and marked atrophy under microgravity and its simulated conditions. Studies using non-weight-bearing animals have provided evidence that increased protein breakdown is, at least in part, responsible for the muscle atrophy (Goldspink et al., 1986; Thomason et al., 1989; Tischler et al., 1990). We previously reported that spaceflight (STS-90) as well as tail-suspension enhanced degradation of rat myosin heavy chain (MHC) in association with activation of ubiquitin-proteasome pathway (Ikemoto et al., 2001). The serial studies showed that oxidative stress may play an important role in triggering ubiquitination of skeletal muscle protein caused by tail-suspension, and that cysteine supplementation prevented ubiquitination and degradation of muscle protein in association with redox regulation in the muscle (Ikemoto et al., 2002). Based on these findings, we suggest that supplementation of antioxidative nutrients may be beneficial to unweighting-induced muscle atrophy.

Vitamin E (α -tocopherol) is a potent antioxidative nutrient, which has been reported to decrease the rate of muscle atrophy induced by immobilization (Kondo et al., 1992; Appell et al., 1997). Furthermore, Reznick et al., (1992) has reported that a 4-wk-feeding with a high α -tocopherol diet (10,000 IU/kg diet=100~150 mg/rat/day) substantially prevented exercise-induced protein oxidation in skeletal muscle. With their experimental regimen and dosage of the administered vitamin E, its concentration in muscle tissue increased three-to five-fold to act well as a radical scavenger. In contrast, 4-wk-feeding of vitamin E (10,000 IU/kg diet) has been reported

Keywords: antioxidative nutrients, ubiquitin, myosin heavy chain, tail-suspension, α -tocopherol, rats

not to attenuate free radical-mediated muscle injury in rats with an eccentric exercise (Warren et al., 1992). Thus, its preventive effects on muscle atrophy or injury is still unclear.

In this study, we examined effects of vitamin E on hindlimb muscle mass loss caused by tail-suspension. Vitamin E did not prevent suspension-induced protein ubiquitination and MHC fragmentation as well as muscle mass loss. The measurement of parameters for oxidative stress, such as concentrations of glutathione (GSH), glutathione disulfide (GSSG) and thiobarbituric acid-reactive substance (TBARS), in skeletal muscle of tail-suspended rats revealed that daily supplementation of a relative high dose (15 mg=15 IU/rat) of α -tocopherol acted as a prooxidant to tail-suspended rats: thereby it may have no preventive effect on suspension-induced muscle mass loss.

Methods

Tail-suspension and vitamin E supplementation

Six-wk-old male Wistar rats were subjected to tail-suspension as described previously (Ikemoto et al., 2001; Ikemoto et al., 2002). Their tails were suspended to keep their hind legs off the ground. Rats were housed in a room maintained at 23°C on a 12-hr light/dark cycle and were allowed free access to a 20% casein diet containing normal level of α -tocopherol and water. Food intake of tail-suspended rats decreased to about 80% of that of non-suspended rats. Pair-fed control rats without suspension were prepared for the same duration. Vitamin E-free corn oil (vehicle) from Funabashi Farm Park, Chiba, Japan, and 1.5 or 15 mg of α -tocopherol acetate, a kind gift from Eisai Co., Tokyo, Japan, in 250 μ l of vehicle were intragastrically delivered to tail-suspended rats every day from the start of tail-suspension (Day 0), at most, for 3 wks. The gastrocnemius muscle was immediately isolated and frozen in liquid nitrogen after finishing suspension. The pair-fed, non-suspended rats were treated with 250 μ l of vehicle for the same period.

All of the treatments of rats were performed according to the Guide for the Care and Use of Laboratory Animals (1985) and approved by the Animal Care Committee of National Space Development Agency of Japan (NASDA) counterpart.

Measurement of GSH, GSSG and TBARS levels in skeletal muscle

Concentrations of GSH and GSSG in gastrocnemius muscle were determined by a high performance liquid column chromatography (HPLC) system according to the method of Reed et al. (1980). Frozen and then pulverized muscle (50 mg) was homogenized with 0.2 ml of ice-cold 5% perchloric acid. The homogenate was placed on ice for 30 min. After the mixture was centrifuged at 11,300 $\times g$ for

20 min at 4°C, sulfhydryl groups of proteins in the supernatant were iodoacetylated: the supernatant mixed with 130 mM iodoacetate and excess of NaHCO₃ was incubated in the dark for 20 min at room temperature. 1-Fluoro-2,4-dinitrobenzene (1.5%) was added to the solution and it was placed at 4°C overnight in the dark. The fluorodinitrophenyle sample was subjected to a reverse-phase column (Spherisorb-5NH₂, 4.6 \times 250 mm, Chemco, Tokyo, Japan) in a HPLC system equilibrated with 80% methanol. The column was washed and developed with a linear gradient of 0.7–3.4 M acetate buffer, pH 4.5 in 80% methanol. GSH and GSSG were eluted with 3.3 and 3.4 M acetate buffer, respectively.

Concentration of TBARS in gastrocnemius muscle was measured by the fluorometric method of Ohkawa et al. (1979). Briefly, the frozen gastrocnemius muscle was homogenized for 1 min on ice with a Polytron homogenizer in nine volumes of ice-cold 1.15% KCl. After the unbroken tissue was removed, 0.2 ml of the homogenate was mixed with 3.8 ml of 8% acetic acid buffer, pH 3.5, containing 0.4% sodium dodecyl sulfate (SDS), 0.03% butylated hydroxytoluene and 0.3% TBA. The mixture was boiled at 95°C for 60 min. After it was cooled with tap water, 1.0 ml of distilled water and 5.0 ml of *n*-butanol and pyridine solution (15:1, v/v) were added. The mixture was vortexed vigorously and centrifuged at 2,000 $\times g$ for 10 min at room temperature. Fluorescence (λ_{ex} =515 nm, λ_{em} =553 nm) in the organic layer was measured. Tetraethoxypropane was used as an external standard. The level of TBARS was expressed as an equivalent of malondialdehyde.

Western blot analysis

Western blot analyses for ubiquitinated proteins and MHC were performed as described previously (Ikemoto et al., 2001). The frozen muscle was homogenized in three volumes of 20 mM Tris-HCl buffer, pH 7.5, containing 5 mM EDTA, 10 mM dithiothreitol, 10 μ M epoxomicin (Peptide Institute, Osaka, Japan) and a protease inhibitor cocktail set (Roche Diagnostics, Tokyo, Japan). After centrifugation at 30,000 $\times g$ for 30 min at 4°C, proteins (40 μ g/lane) in the supernatant (soluble fraction) were subjected to SDS-6%-polyacrylamide gel electrophoresis (PAGE) and transferred to a polyvinylidene difluoride membrane at 35 mA for 6 hr at 4°C. The membrane was blocked with 3% skim milk and then incubated for 1 hr at 25°C in phosphate-buffered saline (PBS) with a 1:500 dilution of a polyclonal antibody against bovine ubiquitin (Sigma, St. Louis, MO, USA) or monoclonal antiserum against rabbit skeletal fast-type MHC (Sigma). The bound antibodies were detected by using the enhanced chemiluminescence system (Amersham, Little Chalfont, England, UK).

Immunohistochemical analysis

Gastrocnemius muscle was fixed with formalin in PBS for 6 hr at room temperature. The muscle was dehydrated and embedded in paraffin wax. Section (6 μ m) was cut and air-dried for 2 hr at room temperature. After dewaxing with xylene, the section was washed with ethanol and rehydrated with distilled water. The section was incubated in 0.3% hydrogen peroxide in PBS for 30 min. After blocking unspecific binding sites with non-immunized rabbit sera for 20 min in a humidified chamber, the section was incubated in a 1:500 dilution of a polyclonal antibody against bovine ubiquitin (Sigma) for 1 hr at 25°C. Bound antibodies were visualized with a three-stage indirect immunoperoxidase kit (Dako Japan, Kyoto), using 3,3'-diaminobenzidine (Sigma) as a substrate.

Proteasome activity

Proteasome activity in the soluble fraction was measured as succinyl-Leu-Leu-Val-Tyr-7-(4-methyl)-coumarylamide-hydrolyzing activity in the presence of 0.05% SDS, according to the method of Ugai et al. (1993). Protein concentrations were measured by Lowry's method by using bovine serum albumin as a standard.

Statistical analysis

All data are expressed as mean \pm SD and were statistically evaluated by analysis of variance (ANOVA) with SPSS software (release 6.1; SPSS Japan Inc., Tokyo, Japan). One-way ANOVA was used to determine the

significant effects of tail-suspension and administration of vitamin E on the measured variables. Individual differences between groups were assessed using Duncan's multiple range test. The differences were considered significant at $P < 0.05$.

Results

No preventive effect of vitamin E on hindlimb muscle mass loss caused by tail-suspension

Tail-suspension of rats for 10 days or longer caused mass loss of gastrocnemius and soleus muscles (Fig. 1), as described previously (Ikemoto et al., 2001). Daily treatment with 1.5 or 15 mg/rat of vitamin E for 3 wks did not prevent the suspension-induced hindlimb muscle mass loss, unlike that of cysteine (Fig. 1 and Ikemoto et al., 2002). To elucidate the mechanism of the non-effectiveness of vitamin E, we further examined oxidative stress markers, ubiquitination of muscle proteins and MHC fragmentation in gastrocnemius muscles of rats treated with 15 mg of α -tocopherol, as described below.

Enhanced oxidative stress in skeletal muscle of tail-suspended rats treated with vitamin E

In consistent with our previous report (Ikemoto et al., 2002), tail-suspension decreased GSH level and reciprocally accumulated GSSG in rat gastrocnemius muscle (Fig. 2A and B). In vitamin E-treated and suspended rats, remark oxidative stress in skeletal muscle

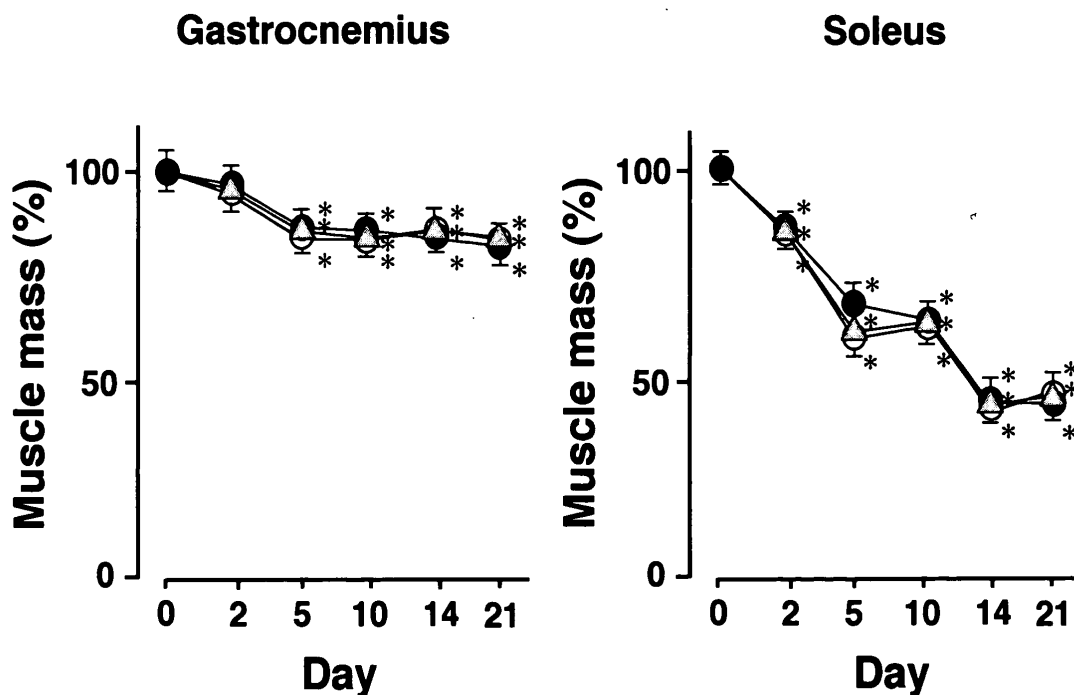


Fig. 1 Effects of vitamin E supplementation on hindlimb muscle mass in tail-suspended rats. Rats were suspended and daily injected with a vehicle (○), and 1.5 (▲) or 15 mg/rat (●) of vitamin E for 3 wks. The pair-fed, non-suspended rats were treated with 250 μ l of vehicle for the same period. Rats were killed on the indicated day and hindlimb muscle wet mass standardized by the body weight was measured. Values of the pair-fed, non-suspended rats are shown as 100%. Values are expressed as mean \pm SD ($n=5$). Means with different superscripts are significantly different from the values on Day 0 at $P < 0.05$.

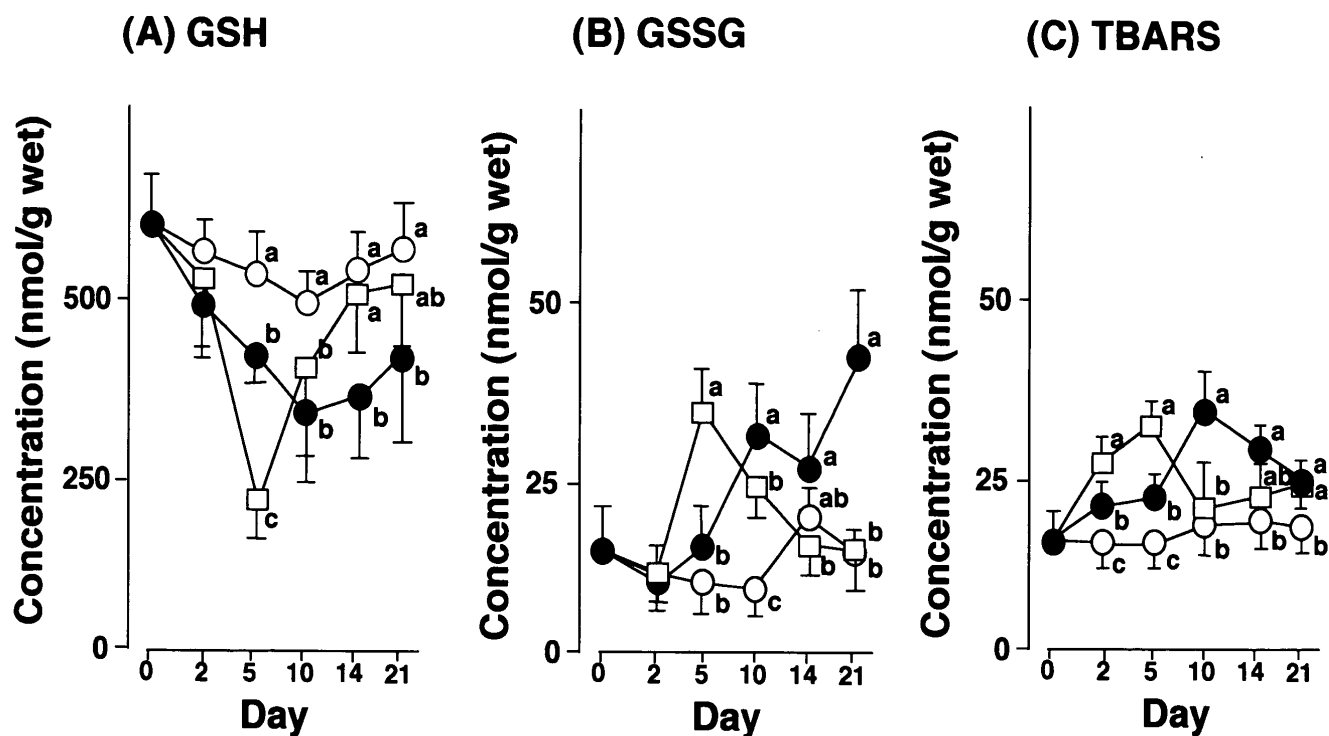


Fig. 2 Changes in concentrations of GSH, GSSG and TBARS in gastrocnemius muscle of tail-suspended rats treated with vitamin E. Vehicle (●) or 15 mg of vitamin E (□) were intragastrically delivered to tail-suspended rats every day from the start of tail-suspension (Day 0). The pair-fed, non-suspended rats were treated with vehicle (○). The gastrocnemius muscle was removed on the indicated day, and concentrations of GSH (A), GSSG (B) and TBARS (C) were immediately measured. Values are mean \pm SD (n=5). Means with different superscripts are significantly different at $P < 0.05$.

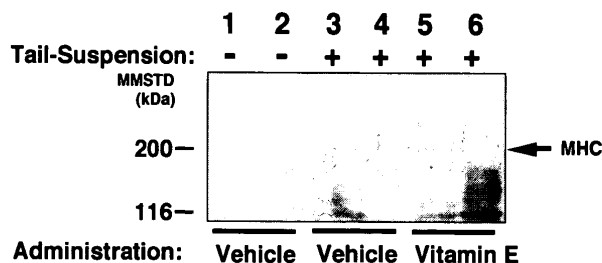
was observed on Day 5, while it restored to basal redox status from Day 14 to 21 (Fig. 2A and B). The supplementation of vitamin E did not prevent accumulation of oxidized lipids in the muscle (Fig. 2C). In addition, concentrations of GSSG and TBARS in vitamin E-treated, suspended rats reached their peak values earlier than those in vehicle-treated, suspended rats.

Effects of vitamin E on protein ubiquitination caused by tail-suspension

Tail-suspension of rats for 10 days or longer also caused ubiquitination of muscle proteins with higher molecular masses (200–350 kDa), including MHC, as described previously (Ikemoto et al., 2001). Daily treatment with 15 mg/rat of vitamin E for 5 days, when oxidative stress reached its peak, induced ubiquitination of muscle proteins with molecular masses from 200 to 350 kDa, whereas the ubiquitination of those proteins was hardly observed in 5-day-vehicle-treated and suspended (control) rats (Fig. 3A). A 200-kDa ubiquitinated protein was immunoreactive to an antibody against rabbit skeletal fast-type MHC (data not shown), as described previously (Ikemoto et al., 2001). Two- and three-wk-treatment with 15 mg/rat of vitamin E caused protein ubiquitination in the muscle of tail-suspended rats with similar extent to those of the respective control rats (Fig. 3B and data not shown).

Immunohistochemical analysis for ubiquitinated

(A) 5-day-tail-suspension



(B) 21-day-tail-suspension

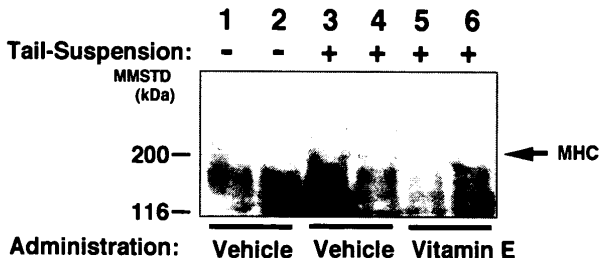


Fig. 3 Effects of vitamin E supplementation on protein ubiquitination in gastrocnemius muscle of tail-suspended rats. Sample was prepared from gastrocnemius muscle of unweighting rats treated with a vehicle or vitamin E (15 mg/rat) for 5 (A) or 21 days (B). Proteins (40 μ g/lane) were subjected to SDS-6% PAGE and transferred to a polyvinylidene difluoride membrane. Ubiquitinated proteins were detected as described in Methods. Similar results were obtained in three separate experiments. MMSTD represents molecular masses of standards.

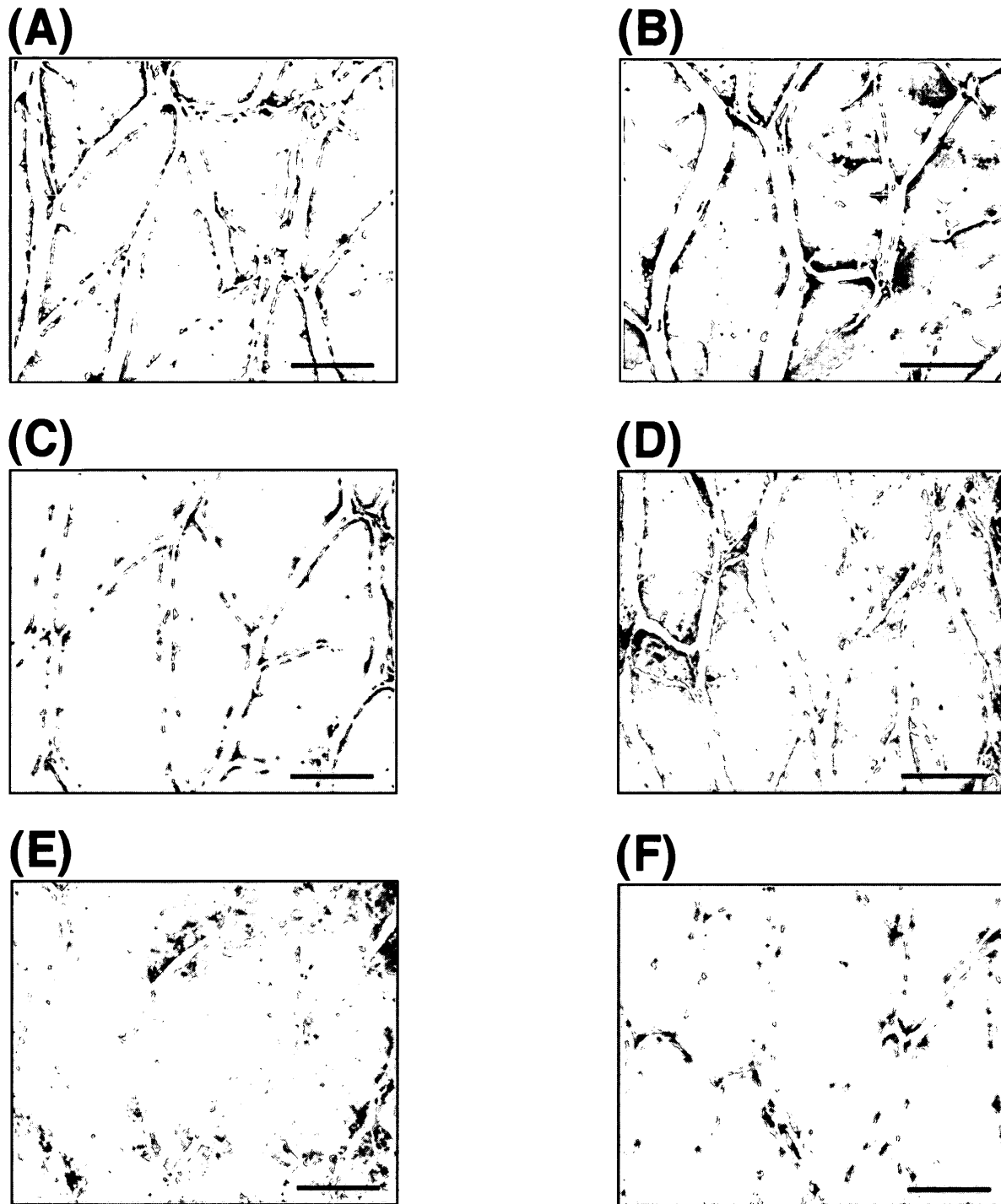


Fig. 4 Histochemical analysis for ubiquitination in gastrocnemius muscle of tail-suspended rats. Rats were suspended and treated with a vehicle (A and D) or 15 mg/rat of vitamin E (B and E) for 5 days and 3 wks, respectively. The pair-fed, non-suspended rats were also treated with 250 μ l of vehicle for 5 days and 3 wks (C and F). Section (6 μ m) of gastrocnemius muscle was prepared and subjected to immunohistochemical analysis of protein-ubiquitination as described in Methods. The length of the bar indicates 100 μ m.

proteins also revealed that supplementation of vitamin E for 5 days caused muscle protein ubiquitination, compared with that of vehicle-treated, suspended rats (Fig. 4A and B). In contrast, protein ubiquitination was observed at the similar extent to that of control rats in gastrocnemius muscle of rats treated with vitamin E for 3 wks (Fig. 4D and E).

Effects of vitamin E on MHC fragmentation and proteasome activity in tail-suspended rats

We previously reported that the immunoreactive proteins corresponding to the MHC degradation products had significantly accumulated by day 10 and continued to increase in tail-suspended rats (Ikemoto et al., 2001). MHC fragmentation was not observed in gastrocnemius muscle of unweighting rats treated even with vitamin E for

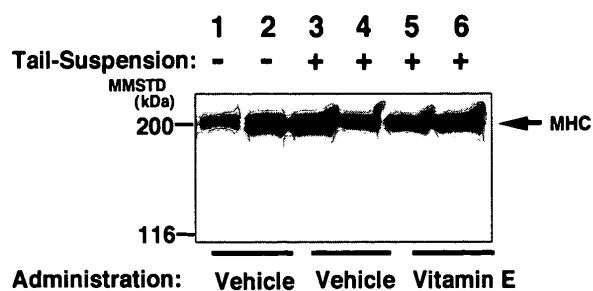
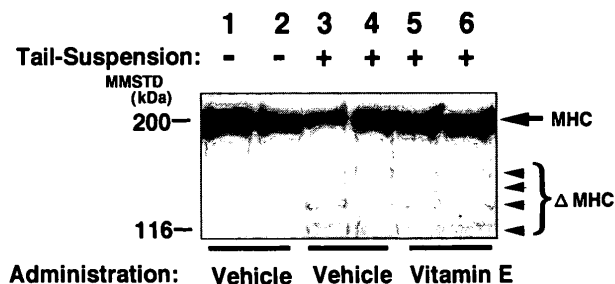
(A) 5-day-tail-suspension**(B) 21-day-tail-suspension**

Fig. 5 Effects of vitamin E supplementation on MHC fragmentation in gastrocnemius muscle of tail-suspended rats. Sample was prepared from gastrocnemius muscle of unweighting rats treated with a vehicle or vitamin E (15 mg/rat) for 5 (A) or 21 days (B). Proteins (40 μ g/lane) were subjected to SDS-6%-PAGE and transferred to a polyvinylidene difluoride membrane. MHC (arrows) and its fragments (Δ MHC, closed triangles) were detected as described in Methods. Similar results were obtained in three separate experiments. MMSTD represents molecular masses of standards.

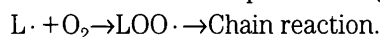
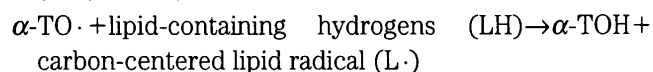
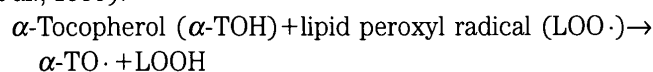
5 days (Fig. 5A), although the vitamin E treatment quickly induced muscle protein ubiquitination, including MHC on Day 5. Tail-suspension for 21 days significantly caused MHC fragmentation in the muscle and vitamin E supplementation did not prevent it (Fig. 5B). In addition, vitamin E supplementation for 5 and 21 days did not change muscle proteasome activity, which was increased by tail-suspension: proteasome activities of unweighting rats treated with vitamin E for 5 and 21 days were 0.71 ± 0.06 and 1.31 ± 0.10 mIU/mg protein (mean \pm SD, $n=3$), respectively, and those of the respective control rats were 0.73 ± 0.05 and 1.30 ± 0.09 mIU/mg protein.

Discussion

To investigate mechanism of non-effectiveness of vitamin E (15 mg/rat) on the muscle mass loss, we measured the redox status (the ratio of GSH to GSSG) in gastrocnemius muscle of vitamin E-treated and suspended rats. Unexpectedly, the vitamin E supplementation decreased the redox status in the muscle of unweighting rats earlier than the vehicle treatment did. Furthermore, the supplementation of vitamin E also accumulated oxidized lipids in the muscle in the early stage (Fig. 4C). These findings indicate that vitamin E at this dose may

function as a prooxidant rather than an antioxidant in tail-suspended rats leading us to consider that the prooxidant action of vitamin E may, at least in part, be responsible to its non-effectiveness.

Several lines of investigations have suggested that α -tocopherol may act as a prooxidant *in vitro* and *in vivo* when co-antioxidants are exhausted under a condition of mild oxidation (Terao and Matsushita 1986; Kontush et al., 1996; Upston et al., 1999). It was likely that tail-suspension induced mild oxidation especially at its early stage, judging from the results as to protein ubiquitination in rat myoblastic L6 cells treated with 0.5 mM H_2O_2 (our unpublished data). Therefore, vitamin E administered here might enhance further lipid peroxidation in skeletal muscle as shown in the following chain reaction (Upston et al., 1999):



Further examinations are required to elucidate this hypothesis.

We suggest that oxidative stress may trigger ubiquitination of muscle protein caused by unweighting (Ikemoto et al., 2001; Ikemoto et al., 2002). Vitamin E supplementation aggravated oxidative stress in skeletal muscle only Day 5 to Day 10 and restored muscular concentrations of GSH, GSSG and TBARS to the basal levels on Day 14 and later. Muscle protein ubiquitination stated on Day 5, where oxidative stress reached its peak, and retained its level for the tested period. Oxidative stress occasionally causes irreversible structural alterations of proteins, subsequently leading to ubiquitination (see Jentsch et al., 1991, for review). Therefore, vitamin E could not prevent protein ubiquitination by tail-suspension in this case, even though it suppressed oxidative stress at the late stage of tail-suspension. In contrast, vitamin E supplementation did not enhance MHC fragmentation and hindlimb skeletal muscle mass loss caused by tail-suspension, while it significantly stimulated ubiquitination of muscle proteins, including MHC. At present, detail mechanism of this discrepancy is unknown. Several lines of investigations suggest that ubiquitination is not always accompanied with activation of proteasome under oxidative conditions (Reinheckel et al., 1998). Concomitantly, vitamin E supplementation did not change muscle proteasome activity in tail-suspended rats in the present study.

We here used a relative high dose of vitamin E: 15 mg/rat of α -tocopherol was corresponded to approximately one-tenth of dose of vitamin E which sufficiently inhibited protein oxidation in skeletal muscle of exercised rats (Reznick et al., 1992). However, even this dosage of vitamin E was too high to function as an

antioxidant in tail-suspended rats. In an effective case of vitamin E supplementation to immobilization-induced muscle atrophy, vitamin E was daily administered at the concentration of 30 mg/kg body weight (≈ 6 mg/rat) for 6 days before immobilization (Kondo et al., 1992). However, daily treatment with 1.5 mg/rat of vitamin E for 3 wks also failed to prevent suspension-induced decline in hindlimb muscle mass. Further examinations are necessary to determine dosages of vitamin E effective to unweighting-induced muscle mass loss.

Acknowledgments We thank T. Hoshino, Eisai Co. (Tokyo, Japan) for a kind gift of α -tocopherol acetate. We are also grateful to J. Terao, Division of Food Science, The University of Tokushima (Tokushima, Japan) for his kind and critical advice to complete this work. This study was supported by Grants-in-aid of "Ground Research Announcement for Space Utilization" promoted by NASDA and Japan Space Forum to T. N.

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Received: May 21, 2001

Accepted: August 1, 2002

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